VOLUME 1 JUNE 1955 NUMBER 6

Canadian Journal of Microbiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MICROBIOLOGY

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Canadian
Journal of Microbiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 1 JUNE 1955 NUMBER 6

UTILIZATION OF SODIUM CASEINATE BY *LACTOBACILLUS CASEI*[1]

BY I. J. MCDONALD AND N. E. GIBBONS

Abstract

Unhydrolyzed sodium caseinate, sodium α-caseinate, and sodium β-caseinate were utilized as sources of amino acids by five strains of *Lactobacillus casei*. Cysteine stimulated utilization of these proteins and, in its presence, the amount of growth was essentially the same with unhydrolyzed or enzymatically hydrolyzed sodium caseinate.

Introduction

Although lactobacilli are known to require complex mixtures of amino acids (6) or casein hydrolyzates (11) for growth, direct utilization of unhydrolyzed protein has not been demonstrated clearly. Previous studies showed that growth of some species in whey (9) and milk serum (8) was enhanced in the presence of sodium caseinate and suggested that casein was utilized. However, since the initial growth in these media was supported by soluble nonprotein nitrogen, the increased growth may have been due to hydrolysis of the caseinate by liberated endoproteinases of these organisms (1, 2). Recent reports that a strain of *Lactobacillus bulgaricus* required a protein from whey or liver for growth in an otherwise complete medium (14) and that unhydrolyzed casein had strepogenin activity for a strain of *Lactobacillus casei* (4) indicate the direct utilization of unhydrolyzed protein by lactobacilli. The present investigation was undertaken to determine the factors affecting utilization of unhydrolyzed sodium caseinate as a source of amino acids for *Lactobacillus casei*.

Materials and Methods

Sodium Caseinate

Raw milk of low bacterial count and less than four hours old was separated at 16° C. Isoelectric casein was precipitated by acidifying the skim milk at 2° C. to pH 4.5 with cold hydrochloric acid. The whey was siphoned off and the casein washed six times with distilled water at 4° C. (7). The casein

[1] *Manuscript received February 11, 1955.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3600.

[The April number of this Journal (Can. J. Microbiol. 1 : 293-363. 1955) was issued April 5, 1955.]

was then redissolved in cold dilute sodium hydroxide (0.1 *N*), reprecipitated with hydrochloric acid, and finally redissolved in cold dilute sodium hydroxide to give a 6% solution. The sodium caseinate (pH 6.8) was shell frozen and dried under vacuum. Sodium caseinate solutions (2.0 gm./100 ml.) containing *ca.* 2.70 mgm. nitrogen/ml. and *ca.* 0.026 mgm. NPN (trichloroacetic acid soluble nitrogen)/ml. were sterilized by Seitz filtration. The molecular weight of the sodium caseinate was approximately 70,000 as determined in 0.2 *M* sodium chloride by the osmotic pressure method.

Alpha and Beta Casein

Purified α-casein or β-casein* was dissolved in dilute sodium hydroxide or 0.1 *M* phosphate buffer (pH 6.8) and sterilized by Seitz filtration. The α-caseinate solutions contained *ca.* 2.20 mgm. nitrogen/ml. and *ca.* 0.010 mgm. NPN/ml.; the β-casein solutions contained *ca.* 1.90 mgm. nitrogen/ml. and *ca.* 0.012 mgm. NPN/ml.

Beta Lactoglobulin

Two per cent solutions of crystalline β-lactoglobulin (Pentex Incorporated, Kankakee, Ill.) in 0.9% sodium chloride (pH 6.8) containing *ca.* 3.00 mgm. nitrogen/ml. and *ca.* 0.040 mgm. NPN/ml. were sterilized by Seitz filtration.

Bovine Serum Albumin

Solutions of crystalline bovine serum albumin (Armour) were prepared in the same manner as β-lactoglobulin. Nitrogen contents were not determined.

Cultures

The strains of *Lactobacillus casei* were: *BC-1*, from the Department of Bacteriology, University of Wisconsin, *L-1*, from the Prairie Regional Laboratory, N.R.C., Saskatoon, Sask., *B-49* and *B-50*, from the Department of Dairying, University of British Columbia, and *7469* from A.T.C.C. Stock cultures were transferred weekly in milk and held at 4° C. between transfers. The classification of the cultures was verified by determining the ability to ferment carbohydrates, the percentage of lactic acid produced in milk, the rotation of the lactic acid, and the maximum and minimum temperatures of growth (12). Strains *BC-1*, *L-1*, and *7469* grew at 45° C., strains *B-49* and *B-50* did not.

Inoculum

Cells used as inocula were from second serial subcultures in basal medium (Table I) supplemented with Bacto-casitone (0.1%), Bacto-casamino acids (0.1%), and Bacto-yeast extract (0.1%). After 18 to 24 hr. incubation at 35° C., the cells were centrifuged, washed three times, and diluted at 10 times their original volume in 0.9% saline. An inoculum of 0.1 ml. was used per 10 ml. of medium.

* *Purified α-casein and β-casein were obtained through the courtesy of Dr. T. L. McMeekin, Eastern Regional Research Laboratory, U.S. Department of Agriculture, Philadelphia, Pa.*

TABLE I

BASAL MEDIUM
Amounts per 5 ml. double strength medium

	mgm.		μgm.
Lactose	200	Thiamine hydrochloride	4.0
Sodium acetate	200	Pyridoxal hydrochloride	4.0
K_2HPO_4	5.0	Pyridoxamine dihydrochloride	4.0
KH_2PO_4	5.0	*p*-Aminobenzoic acid	4.0
$MgSO_4.7H_2O$	2.0	Riboflavin	8.0
$MnSO_4.H_2O$	0.4	Nicotinic acid	8.0
$FeSO_4.7H_2O$	0.1	Calcium pantothenate	8.0
Adenine sulphate	0.2	Folic acid	0.2
Guanine hydrochloride	0.2	Biotin	0.04
Uracil	0.2		
Asparagine	2.0	pH 6.0	
Tween 80	10.0		

Experimental Procedure

Basal medium was autoclaved at 15 lb. pressure for 15 min. Seitz filtered solutions of cysteine and other reducing agents and of sodium caseinate and other proteins were added to the cooled medium, and the volume adjusted with sterile distilled water. After inoculation and incubation at 35° C., growth was determined by titration of the acid produced by the test organism and was expressed as per cent titratable acidity calculated as lactic acid.

Results

In basal medium without asparagine and with unhydrolyzed sodium caseinate as the sole source of amino acids, *L. casei* strains showed limited growth (Table II). Dialysis of the sodium caseinate or treatment with activated carbon (at pH 6.8) did not alter the results. Growth was not supported by basal medium containing filtrates obtained by precipitation of

TABLE II

EFFECT OF REDUCING AGENTS ON UTILIZATION OF SODIUM CASEINATE BY *Lactobacillus casei*
(54 hr. at 35° C.)

Additions to medium* (4 μM./ml.)	Titratable acidity as % lactic acid			
	Strain			
	BC-1	L-1	B-49	B-50
None	0.07	0.34	0.14	0.11
Cysteine	1.65	1.58	0.25	0.25
Thioglycolate	1.09	1.04	0.19	0.21
Ascorbic acid	0.23	0.56	0.10	0.13

* *Basal medium (Table I) without asparagine, plus sodium caseinate (2.0 mgm./ml.). Reducing agents added as Seitz filtered solutions.*

casein from sodium caseinate solutions, and growth in basal medium plus sodium caseinate was not stimulated by the addition of these filtrates. It appeared therefore, that the nonprotein nitrogen of the sodium caseinate solutions had little effect on the growth of the organisms.

When reducing agents were added to the caseinate medium, growth was stimulated (Table II). Cysteine was effective with the four strains tested. Thioglycolate was less effective than cysteine and ascorbic acid was less stimulatory than thioglycolate. Strains BC-1 and L-1 showed greater response than B-49 and B-50; ascorbic acid was inactive with the latter two strains.

Growth of *L. casei* B-49 and B-50 was relatively poor in the above medium even in the presence of cysteine. The addition of asparagine (0.4 mgm./ml. basal) increased the growth of these strains without affecting the growth of the others. Asparagine, therefore, was incorporated in the basal medium for all further experiments.

TABLE III

EFFECT OF HOMOCYSTEINE AND METHIONINE ON UTILIZATION OF SODIUM CASEINATE BY *Lactobacillus casei*
(43 hr. at 35° C.)

Additions to medium* (0.1 μM./ml.)	Titratable acidity as % lactic acid			
	Strain			
	BC-1	L-1	7469	B-49
None	0.32	0.23	0.10	0.02
Cysteine	0.92	0.86	0.70	0.31
Homocysteine	0.34	0.39	0.34	0.09
Methionine	0.16	0.07	0.18	0.02

* *Basal medium (Table I) plus sodium caseinate (2.0 mgm./ml.).*

TABLE IV

EFFECT OF INCUBATION UNDER HYDROGEN ON UTILIZATION OF. SODIUM CASEINATE BY *Lactobacillus casei*
(46 hr. at 35° C.)

Additions to medium*	Atmosphere	Titratable acidity as % lactic acid		
		Strain		
		BC-1	L-1	B-49
None	Air	0.04	0.09	0.34
Cysteine	Air	0.90	1.04	0.58
None	Hydrogen	0.04	0.11	0.31
Cysteine	Hydrogen	0.72	1.01	0.46

* *Basal medium (Table I) plus sodium caseinate (2.0 mgm./ml.).*

Other sulphydryl compounds were tested for their stimulation of *L. casei*. No detectable difference was found in the effects produced by cysteine, cystine, or glutathione (reduced). Homocysteine and methionine were less stimulatory than cysteine (Table III) but homocysteine was more active than methionine. Other trials (results not shown) indicated that mercaptosuccinic acid was slightly stimulatory while 2,3-dimercaptopropanol was not. It was shown also that the cysteine effect could not be obtained by incubation of cultures under hydrogen (Table IV).

Strains of *L. casei* grew slowly with sodium α-caseinate or sodium β-caseinate as the sole source of nitrogen (Table V, Expt. I) and cysteine stimulated growth. In the presence of cysteine, one strain, *L. casei* B-49, utilized α-casein more readily than β-casein; the remainder preferred β-casein and the total growth was about equal to that with sodium caseinate.

When an enzymatic hydrolyzate of sodium caseinate was supplied as a source of amino acids, good growth was obtained (Table V, Expt. II). Stimulation of growth by cysteine was considerably less with hydrolyzed casein than with unhydrolyzed casein. Also, growth of the organisms in the

TABLE V

UTILIZATION OF ALPHA AND BETA CASEIN BY *Lactobacillus casei*
(At 35° C.)

	Titratable acidity as % lactic acid							
	Strain							
	BC-1		L-1		7469		B-49	
Additions to medium*	45 hr.	96 hr.	45 hr.	96 hr.	45 hr.	96 hr.	45 hr.	96 hr.
Experiment I								
Na α-caseinate	0.07	0.23	0.11	0.23	0.07	0.18	0.07	0.14
Na α-caseinate + cysteine	0.13	0.54	0.14	0.40	0.08	0.27	0.33	0.65
Na β-caseinate	0.04	0.04	0.06	0.11	0.05	0.16	0.07	0.09
Na β-caseinate + cysteine	1.06	1.75	1.06	1.66	1.46	1.51	0.08	0.13
Na caseinate	0.05	0.13	0.07	0.11	0.06	0.15	0.06	0.11
Na caseinate + cysteine	1.30	1.69	0.91	1.69	1.19	1.69	0.36	1.10
Experiment II								
Hydrolyzed Na caseinate†	1.37	—	0.88	—	1.03	—	—	—
Hydrolyzed Na caseinate + cysteine	1.42	—	0.98	—	1.15	—	0.70	—
Experiment III								
Hydrolyzed Na α-caseinate† + cysteine	1.29	—	1.49	—	1.28	—	0.70	—
Hydrolyzed Na β-caseinate† + cysteine	1.06	—	1.45	—	1.24	—	0.36	—

** Basal medium (Table I) + caseinate (2.0 mgm./ml.) and cysteine (0.1 μM./ml.) when added.*

† Basal medium plus hydrolyzate equivalent to 2.0 mgm./ml. of caseinate solution. Hydrolyzates prepared by adding pancreatin (0.2 mgm./ml.) to caseinate solutions (2.0 gm./100 ml.) at pH 6.8 and incubating at 30° C. for 24 hr.

presence of cysteine was about the same when the nitrogen source was either hydrolyzed or unhydrolyzed sodium caseinate. In the presence of cysteine, strains of *L. casei* grew well (Table V) in either hydrolyzed α- or β-casein. Strain B-49 utilized the α-casein hydrolyzate more readily than the β-casein hydrolyzate but the other strains used each hydrolyzate equally well.

To determine if other proteins were utilized by *L. casei*, unheated β-lactoglobulin and bovine serum albumin were supplied as nitrogen sources in basal medium plus cysteine. Growth was not obtained with these proteins or with β-lactoglobulin heated at 121° C. for 15 min. In media containing cysteine and pancreatin hydrolyzate of autoclaved β-lactoglobulin, however, the organisms grew well, producing about one-half the amount of acid produced in hydrolyzed caseinate medium under the same conditions.

Discussion

Growth of *Lactobacillus casei* with unhydrolyzed sodium caseinate indicates the presence of proteinase in the medium. This enzyme system appears to be activated by cysteine and, in this respect, is similar to previously described endoproteinases of lactobacilli (1, 2). Since neither serum albumin nor β-lactoglobulin supported growth, it would appear that these proteins were not hydrolyzed by this enzyme system. Thus the enzyme also resembles trypsin which has been reported to hydrolyze casein but not the native proteins ovalbumin and pseudoglobulin (5).

Differences in the ability of strains of *L. casei* to utilize α- and β-casein is probably related to differences in enzyme systems of the organisms. Previous work showed that α- and β-casein react differently to hydrolysis by microbial enzymes (3, 13) and coagulation by rennin (10). However, since the strain of *L. casei* that preferred unhydrolyzed α-caseinate also grew better with hydrolyzed α-caseinate than with hydrolyzed β-caseinate, it is possible that the ability to utilize these proteins was related also to the nutritional requirements of the organisms. The preferential use of α-casein by strain B-49, as well as its inability to grow at 45° C., may provide an additional diagnostic characteristic for strains of *L. casei* (12).

Acknowledgment

The authors wish to thank Mr. H. W. Mason for valuable technical assistance, Dr. E. O. Hughes for assistance in the preparation of the manuscript, and Dr. M. E. Reichman for the molecular weight determinations.

References

1. AMUNDSTAD, O. En undersøkelse over løpeenzymets og enkelte mjølkesyrebakteriers proteolytiske egenskap med saerlig henblikk på ostens modning. (An investigation of the proteolytic activity of rennin and of some lactic acid bacteria with special reference to cheese ripening). Statens Mejeriförsök, Medd. M. 28, Malmo. 1950.
2. BARIBO, L. E. and FOSTER, E. M. The intracellular proteinases of certain organisms from cheese and their relationship to the proteinases in cheese. J. Dairy Sci. 35 : 149-160. 1952.

3. FRIEDMAN, M. E., NELSON, W. O., and WOOD, W. A. Proteolytic enzymes from *Bacterium linens*. J. Dairy Sci. 30 : 1124-1134. 1953.
4. GYLLENBERG, H., ROSSANDER, M., and ROINE, P. A strain of *Lactobacillus bifidus* which requires strepogenin. J. Gen. Microbiol. 9 : 190-198. 1953.
5. HAUROWITZ, F., TUNCA, M., SCHWERIN, P., and GOKSU, V. The action of trypsin on native and denatured proteins. J. Biol. Chem. 147 : 621-625. 1945.
6. HENDERSON, L. M. and SNELL, E. E. A uniform medium for determination of amino acids with various microorganisms. J. Biol. Chem. 172 : 15-29. 1948.
7. HUNTER, G. J. E. The growth requirements of lactobacilli in relation to cheese flavour development. J. Dairy Research, 17 : 79-90. 1950.
8. MIDDAUGH, P. R. Utilization of nitrogen constituents of milk by *Streptococcus lactis* and *Lactobacillus casei*. Ph. D. Thesis, University of Wisconsin. 1951.
9. ORLA-JENSEN, S., OTTE, N. C., and SNOG-KJAER, A. The vitamin and nitrogen requirements of the lactic acid bacteria. Mém. acad. roy. sci., Danemark, Sect. sci. 9 (Sér 6) : 1-52. 1936.
10. PYNE, G. T. Casein heterogeneity and the rennet coagulation. Chemistry & Industry, 171-172. 1951.
11. ROBERTS, E. C. and SNELL, E. E. An improved medium for microbiological assays with *Lactobacillus casei*. J. Biol. Chem. 163 : 499-509. 1946.
12. ROGOSA, M., WISEMAN, R. F., MITCHELL, J. A., DISRAELY, M. N. Assisted by BEAMAN, A. J. Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius nov. spec.* and *Lactobacillus cellobiosus nov. spec.* J. Bacteriol. 65 : 681-699. 1953.
13. VAN DER ZANT, W. C. and NELSON, F. E. Characteristics of an endocellular proteolytic enzyme system of *Streptococcus lactis*. J. Dairy Sci. 36 : 1212-1222. 1953.
14. WILLIAMS, W. L. and GRADY, J. E. Lactein, a protein required for growth of a Lactobacillus. Federation Proc. 13 : 321-322. 1954.

STUDIES WITH STAPHYLOCOCCAL TOXINS

I. A REAPPRAISAL OF THE VALIDITY OF THE "KITTEN TEST" AS AN INDICATION OF STAPHYLOCOCCAL ENTEROTOXIN[1]

By B. H. Matheson and F. S. Thatcher

Abstract

Under controlled conditions of maintenance and treatment of test cats, and with inducement of decisive vomiting as the criterion of enterotoxicity, the "Dolman kitten-test" has provided a high degree of positivity for the detection of an emetic principle in preparations from staphylococcal cultures. These conclusions are based on 315 individual cat injections. Under the conditions of these experiments and with the use of 133 control preparations, false positive reactions likely to cause confusion in normal determinative tests were not encountered.

The determination of the nature of the enterotoxic principle that is produced by specific strains of staphylococci, and of the mechanism whereby it induces the severe vomiting that is its most conspicuous effect in susceptible animals, has been handicapped by lack of a wholly suitable indicator test or method of assay (23, 25, 26). Human volunteers, rhesus monkeys *(Macaca mullata)*, and young cats have been the preferred test animals to date. Humans and monkeys have been reported to be highly variable in their response to the emetic principle (12, 13, 20, 25) and neither of these subjects is readily available for test purposes.

The suggestion of Robinton (18) that the frog *(Rana pipiens)* is a suitable test animal for staphylococcal enterotoxin has not been endorsed by subsequent workers (10, 23) who have shown that the frog is not sufficiently specific in reaction to be a dependable subject for assay. One of us (F.S.T.) also reached this latter conclusion, based on experiments with 150 frogs. Atypical peristaltic reactions in the stomach or duodenum were observed but not with a degree of constancy and specificity that would allow reliable distinction between enterotoxic and non-enterotoxic preparations.

The "kitten test" as described originally by Dolman, Wilson, and Cockcroft (9) involved intraperitoneal injection of test preparations that had been boiled for 30 min. in order to destroy hemolysins. The young cat continues to be the most commonly chosen test animal (12, 19), in spite of the many criticisms that have been directed against its use.

"False positive" reactions in the cat have been described by a number of investigators. The injection of uninoculated media of the type used for toxin production is reported to have given the same reaction as filtrates from enterotoxigenic cultures (14, 15, 17, 21). Equivocation is also expressed with regard to the role of specific lysins in causing an emetic response (11, 16, 22). On the other hand, the conclusion of Dolman and Wilson (7) and Dolman (6) that no relation exists between the α-lysin or β-lysin and enterotoxin has

[1] *Manuscript received January 26, 1955.*
A contribution from the laboratories of the Food and Drug Directorate Department of National Health and Welfare, Ottawa, Canada.

been upheld by a number of other investigators whose work is discussed by Surgalla and Hite (24). Nevertheless, reviews of the evidence relative to the validity and significance of the kitten test all reveal conflict of opinion and uncertainty (2, 3, 4, 5, 12, 13, 17, 26).

The purpose of this paper, therefore, is to establish under defined conditions to what degree the intraperitoneal injection of cats can provide an indication of the presence or absence of an emetic principle in filtrates of staphylococcus cultures in order to gauge the usefulness of these animals for further studies with toxic preparations from staphylococci.

Methods

In order to minimize the excitability of young cats, all animals to be used for the tests were obtained shortly after weaning and confined to animal cages for a period of three weeks to several months before being used for test purposes. They were maintained on a diet of milk, water, and cooked liver. All cats were under continuous supervision of a veterinarian who approved each animal as in good health before test.

The staphylococcus culture used for the majority of these experiments was a strain of *Micrococcus pyogenes* var. *aureus* isolated in this laboratory from an enterotoxic specimen of cheddar cheese. Using Dolman's medium as substrate for toxin production, this particular strain was found to produce the most potently emetic filtrate from among 80 coagulase-positive isolates of the same species. This strain (L.16) produces α-, β-, and δ-lysins concurrently. The culture was grown at 37° C. in Dolman's medium in an atmosphere of air enriched to 30% carbon dioxide. Filtrates were prepared essentially in accord with the method of Dolman and Wilson (8). Partially purified concentrates of the enterotoxic principle were used on occasion. These were prepared by methods to be described in detail in a later paper. For comparative purposes other enterotoxigenic cultures were used; a subculture from the strain 12069-alpha used by Dolman, an enterotoxigenic strain that produces alpha-lysin but no beta-lysin (6), a strain S.6 used by Surgalla *et al.* (23), which under the conditions of culture described above produced α- and δ-lysins but no β-lysin, and strains 230 and 224 provided by Dr. E. P. Casman[2], which produced no detectable amounts of β- or δ-lysins and only low titers of α-lysin.

Test preparations warmed to 37° C. were slowly injected by the intraperitoneal route into cats about one to two hours after feeding. An interval of at least 10 days was allowed between consecutive injections of the same cat, which would not be used more than four times in order to avoid development of immunity.

The animals used ranged from kittens weighing about 700 gm. to nearly full-grown cats, weighing 3500 gm. Aliquots from test filtrates were injected both before and after boiling for 30 min.

[2] *Dr. E. P. Casman, Food and Drugs Administration, Department of Health, Education and Welfare, Washington 25, D.C.*

A reaction was accepted as positive for an emetic substance if a characteristic sequence of retching followed by decisive vomiting occurred from 10 to 120 min. after injection.

Control preparations consisting of uninoculated media, of reagents and buffers used in the concentration of the toxic factor from culture-filtrates, and filtrates from non-enterotoxigenic strains were tested in identical manner.

A number of specimens that had proved able to cause emesis in cats by intraperitoneal injection were administered per os with the use of catheter tubing and a syringe. Amounts of toxin up to 150 times the normal intraperitoneal dose were fed in this way. In order to protect the toxin from the possibility of becoming inactive by precipitation caused by the acidity of the stomach, some toxin specimens were fed with diluents of milk or buffer solutions.

The specific media tested were: Dolman's medium (8) in which peptone is used as the nitrogenous base; a medium used by Dr. Casman (unpublished)—casein hydrolyzate with tryptophane, sodium acetate, and salts; veal-infusion broth (Difco); "Amigen"—pancreatic digest of casein (Mead Johnson)—plus the non-peptone ingredients of Dolman's medium.

Results

The reaction of cats to injections of boiled enterotoxic preparations followed a typical sequence. Retching and vomiting usually occurred after a lapse of from 15 to 50 min. If the reactions were severe several such episodes took place within about two hours. As many as eleven emetic spasms from a single injection have been noted. The animal would finally rest, refusing food or drink. The following morning it appeared quite normal, was active and hungry.

When toxic filtrates from cultures L.16, 12069 alpha, or S.6 were injected without heating, the emetic reactions were essentially the same as from heated specimens except that the first spasm occurred sooner. Without exception, however, the unheated specimens proved fatal within 12–24 hr. All cats that died from these causes were subjected to post-mortem examination. A typical report from the pathologist follows: "A sero sanguineous fluid was found in the peritoneal cavity. The peritoneum was slightly hyperemic, and a catarrhal inflammation was evident in the mucosal surface of the stomach. The stomach contents were tinged with bile and blood. A condition of mild catarrhal enteritis was noted in the jejunum. The kidneys were hyperemic but the capsule was easily removable. Advanced autolysis in the central nervous system precluded diagnostic observation in these tissues". By contrast, an examination of 10 animals sacrificed after having recovered from the effects of injection of boiled filtrates showed no such condition. "Histopathological examination of the medulla oblongata of an animal that had received a massive dose of an enterotoxic concentrate prepared from a heated filtrate revealed no abnormality that could be detected by the use of the haematoxylin and eosin stain."

Table I reports the occurrence of emesis among 136 cats given intraperitoneal injections of Seitz-filtered preparations from several different culture filtrates, using amounts of filtrate varying from 0.25 ml. to 8 ml. per injection. Of a total of 136 animals, 129 provided positive reactions. Of the seven cats that failed to show a positive emetic reaction, four were naturally resistant (as established by subsequent injection with an amount 10 times greater than had caused a positive reaction in other animals); and one had received an inadequate volume of toxic injectant since aliquots of the same preparation when diluted to larger volume caused characteristic emetic reaction in the same and other animals. The remaining two cats showed several of the incidental signs of impending emesis, but did not reach the point of vomiting.

Table II lists the emetic effect of specific control preparations administered at various volumes. Of 133 cats receiving control preparations only three

TABLE I

EMESIS IN CATS FROM FILTRATES OF *M. pyogenes* (INTRAPERITONEAL ROUTE)

Vol. of filtrate injected (ml.)	No. of cats tested	No. of cats positive	No. of cats negative
0.25	3	2	1
0.5	13	12	1*
0.8	5	5	0
1.0	85	82	3 (2*)
1.5	7	6	1*
2.0	16	15	1
5.0	5	5	0
8.0	2	2	0
Totals	136	129	7

* *Resistant cats.*

TABLE II

EMESIS IN CATS BY CONTROL PREPARATIONS

	No. of cats tested	Volume of injectant (ml.)	No. of cats vomiting
Filtrate: Dolman's medium	31	2–5	0
Casman's medium	3	0.5–5	0
Modified Dolman's media + 1% amigen*	13	0.5–5	0
Veal-infusion broth	5	1–5	0
Distilled water	6	1.5–10	1
Washed precipitate from treated filtrate	5	0.5–1	0
Acetic acid solution (pH 3.8)	40	5–10	1
Citrate-phosphate buffer (pH 7.8)	4	5	0
Filtrate after ppt'n of toxin (dialyzed)	3	1–2	0
Filtrate after ppt'n of toxin (not dialyzed)	3	4–10	0
Crystalline ppt. after removing toxin	3	1–2	0
Filtrate after 24 hr. exposure to filter paper	7	0.5–2	1
Filtrates from non-enterotoxigenic *M. pyogenes aureus*	10	1.0–4	0
	133		3

* *"Amigen"—pancreatic hydrolyzate of casein (Mead Johnson).*

vomited. Each of these vomited only once, whereas it was customary for a preparation from an "enterotoxigenic" culture to cause several emetic spasms in a short period (see Table V). It will be noted that not a single emetic response was obtained from 62 cats that were injected with any of the four different uninoculated media tested. The three control specimens that each caused a single emetic reaction were, respectively, 5 ml. of acetic acid at pH 3.8 (used as an ingredient in a buffer at one point in a purifying procedure), 10 ml. of distilled water, and an aliquot of an originally toxic filtrate in which filter-paper (S and S #598) had been suspended for 24 hr. Only one of 40 cats given the 5 ml. volume of acetic acid reacted. This volume is far in excess of the usual 0.5 ml. of toxic filtrate necessary to cause emesis and the acid might well be an irritant. Similar reasoning would apply also to the injection of 10 ml. of distilled water to which only one of four cats reacted. The third "false-positive" was the sole response from eight cats in an experiment to check the adsorption of enterotoxin on filter paper, and thus might more correctly be considered as other than a control preparation since the result was probably caused by a residuum of unadsorbed toxin.

Table III summarizes the results from injection of redissolved aliquots of lyophilized partially purified concentrates containing an enterotoxic substance from strain L.16, each aliquot being diluted to contain approximately five times the amount of toxin that would be present in the same respective volumes of active filtrate. It will be noted that of the 47 cats tested with such preparations 41 responded positively, and that those specimens that failed to cause emesis were all of small volume (0.25 ml. or less).

Table IV shows the effect of dilution of the same amount of toxin as contained in the negatively reacting small volumes. By diluting the toxin contained in 0.1 ml. or 0.15 ml. to 0.5 ml., all 12 of the tested cats (including those previously negative to 0.1 ml.) reacted positively. The reason for this is obscure. This result led to the preferred use of a minimal volume of 0.5 ml. for further injection tests.

TABLE III

STAPHYLOCOCCUS ENTEROTOXIN—

EMESIS IN CATS FROM ENTEROTOXIC CONCENTRATES*

Vol. injected (ml.)	No. of cats	
	Injected	Positive
0.1	8	4
0.15	3	2
0.25	6	5
0.4	4	4
0.5	18	18
1.0	8	8
Totals	47	41

* *Several different preparations.*

TABLE IV

STAPHYLOCOCCUS ENTEROTOXIN CONCENTRATES—
EFFECT OF VOLUME OF INJECTANT UPON EMESIS

Vol. of concentrate (ml.)	Vol. water added (ml.)	No. of cats	
		Injected	Positive
0.1	0	8	4
0.15	0	3	2
0.1	0.4	7	7
0.25	0.25	5	5

TABLE V

STAPHYLOCOCCUS ENTEROTOXIN—
TIME LAPSE BETWEEN INJECTION AND VOMITING OF CATS—REPRESENTATIVE RESULTS

Specimen	Vol. injected (ml.)	Time lapse after injection (min.)		No. of emetic spasms	Time of death
		First spasm	Last spasm		
Toxic filtrates: unheated					
L.16	0.5	18	60	6	Less than 24 hr.
	0.5	14	108	9	Less than 24 hr.
	1.0	29	82	3	48 hr.
	1.0	23	74	5	Less than 24 hr.
S.6	1.0	26	67	3	Less than 24 hr.
224	1.0	14	83	4	Less than 24 hr.
230	1.0	37	69	3	Survived
Toxic filtrates: boiled 30 min.					
L.16	0.25	13	106	6	Survived
	0.5	23	53	4	Survived
	1.0	15	82	5	Survived
	1.0	22	44	2	Survived
S.6	1.0	43	80	2	Survived
	1.0	26	67	3	Survived
	1.0	42	99	3	Survived
224	1.0	49	106	3	Survived
	1.0	62	94	2	Survived
230	2.0	57	77	2	Survived
	2.0	61	89	2	Survived
Concentrated toxin:* unheated					
S.6 (Dr. Bergdoll's prep'n)	1.0	49	72	3	Less than 24 hr.
Concentrate toxin:* filtrated boiled 30 min.					
S.6	1.0	63	153	3	Survived
	1.0	130	130	1	Survived
L.16	0.25	42	54	2	Survived
	0.5	48	59	2	Survived
	1.0	18	103	7	Survived
	1.0	50	68	4	Survived

* *Diluted 1 : 50 for use.*

The effect upon the cat reaction of boiling either the toxic filtrates or a solution of partially purified toxic concentrates is shown in Table V, which expresses representative results from the 170 positive reactions obtained. With regard to preparations of strain L.16 it may be seen that the number of emetic spasms caused by each injection and the range of time during which emetic spasms occurred was similar whether from heated or unheated preparations. A concentrate from culture S.6 prepared by Dr. M. S. Bergdoll and provided through his kindness was similar to the Ottawa (L.16) concentrate in its reactions, even though prepared by a different method (1).

The median and range in elapsed time between injection and the occurrence of the first emetic spasm for heated and unheated specimens of culture filtrates and of concentrates from five different strains having widely different lytic potentialities are shown in Table VI. The data in this table indicate the similarity in the time necessary to induce emesis by the heated preparations from the various strains, even though each of the three lysins was independently absent from specific filtrates.

Apart from the tendency for an unheated filtrate to cause emesis somewhat more rapidly, the only difference encountered between the comparable heated and unheated specimens was the fact that in every instance throughout this study (Tables V, VI) an unheated toxic filtrate or a concentrated toxic fraction from an unheated filtrate caused death, while a rapid recovery was the consistent sequel to emesis induced by a heated specimen either of filtrate or of concentrate.

Table VII lists the results from experiments designed to cause emesis in cats by the oral route. Of 23 cats that received oral administrations of toxic filtrates not one reacted positively even though up to 150 times the amount

TABLE VI

STAPHYLOCOCCUS ENTEROTOXIN—MEDIAN AND RANGE OF ELAPSED TIME AFTER INJECTION BEFORE FIRST EMETIC SPASM. CATS—INTRAPERITONEAL ROUTE

Specimen	No. of cats tested	Median time lapse (min.)	Range of time lapse (min.)
Boiled filtrates			
12069 alpha (Dolman)	12	38	18–53
224 (Casman)	12	57	30–106
230 (Casman)	2	59	57–61
S6 (Dack *et al.*)	10	47	12–120
L.16 (Thatcher)	100	39	12–91
Unheated filtrate			
L.16	4	17.5	15–91 (lethal)
Concentrates from boiled filtrates (partially purified)			
L.16	64	34	12–76
S.6*	8	72	49–119
Concentrates from unheated filtrates			
S.6*	1	49	One specimen only (lethal)

* *Prepared by Dr. M. S. Bergdoll, Food Research Institute, University of Chicago, Chicago, Ill.*

that was positive by the intraperitoneal route was fed either alone or in milk or in phosphate buffer at pH 7.5. Seventeen of the cats receiving the larger amounts of toxic filtrate frequently passed wet stools and were restless, conditions which are often incident to emesis, but true vomiting was never observed.

TABLE VII

STAPHYLOCOCCUS ENTEROTOXIN—
REACTION OF CATS TO TOXIC FILTRATES* FED BY STOMACH TUBE

No. of cats	Vol. toxic filtrate (ml.)	Diluent added (ml.)		Cat response in 4–6 hr.		
		Milk	Phosphate buffer pH 7.5	Wet stools	Restlessness	Vomiting
1	1	150	—	—	—	—
1	3	150	—	—	—	—
1	3	100	—	—	+	—
1	3	50	—	—	—	—
2	5	5	—	—	—	—
4	10	5	15	—	—	—
2	15	—	15	—	—	—
3	20	—	—	+ (1 cat)	—	—
4	30	—	30	+	+	—
1	40	—	—	+	+	—
2	50	—	—	+	+	—
1	50	—	25	+	+	—
23	—	Totals	—	9	8	0

** 0.5 ml. by intraperitoneal route causes emesis.*

Discussion

The foregoing results show that young cats consistently and characteristically responded to preparations from cultures of five enterotoxigenic strains of staphylococci of widely divergent lytic capacities. "False-positive" reactions from control preparations did not occur under conditions of normal determinative tests. These conclusions are based on 315 animal injections. It is suggested that for most biological tests such results would be considered to have a high order of positivity.

It should be noted that not a single "false-positive" reaction was obtained from 62 preparations from four different uninoculated media. This result differs from a number of reports in the literature even though some of our media were of the same composition as those described as causing false reactions.

The symptoms induced by the boiled filtrates and concentrates from all enterotoxigenic strains were characteristically the same, even though the injectants were from cultures of markedly different lytic potentiality. The symptoms were in general accord with those described by Dolman (6). The

occurrence of death without exception from unheated preparations of lysogenic strains was presumably due to the presence of α- or β-lysins. In our hands, death of a cat has never resulted from a heated preparation.

The conclusion that these results seem to warrant is that with the strains tested in this laboratory and with proper precaution in the pretreatment of test animals, the presence of an emetic principle in a staphylococcus culture can be reliably determined by intraperitoneal injection of cats with heated filtrates essentially in accord with the method of Dolman, Wilson, and Cockcroft (9), provided that decisive vomiting occurs, and provided that the test animals make a prompt recovery.

It is recognized that strains may exist that produce enterotoxins of differing thermolability, just as noted for different preparations of α- and β-lysins (7), though our cultures gave no such indication even though from widely dispersed origins (England; Chicago and Washington, U.S.A.; and Ottawa, Canada). It remains to be shown clearly whether or not an emesis-inducing function for cats may still persist in the molecules of specific lysins even though their capacities to haemolyze blood cells may have been destroyed by heating. Similarly, the extent to which active β-lysin might cause emesis in cats in the absence of an enterotoxin is not known with precision. These aspects will be discussed in later papers.

Acknowledgments

The authors wish to extend thanks to Dr. C. E. Dolman, Dr. G. M. Dack, and Dr. E. P. Casman for the contribution of cultures made valuable by the merit of the researches of their respective donors; to Dr. Dack and his colleagues, Drs. M. J. Surgalla and M. S. Bergdoll, both for the gift of a specimen of enterotoxin partially purified and concentrated by Dr. Bergdoll and for the privilege of helpful discussions on staphylococcal toxins; and to Dr. H. C. Grice for veterinary supervision of our cats and for post-mortem examinations.

The dependable co-operation of our technician, Miss Joan Marshall, was an important factor in this study and is much appreciated.

References

1. BERGDOLL, M. S. Food Research Institute. University of Chicago, Chicago, Ill. Private communication.
2. BLAIR, J. E. The pathogenic staphylococci. Bacteriol. Revs. 3 : 97-146. 1939.
3. CHAPMAN, G. H. An improved stone medium for the isolation and testing of food-poisoning staphylococci. Food Research, 13 : 100-105. 1948.
4. DACK, G. M. Food poisoning. 4th ed. The University of Chicago Press, Chicago, Ill. 1944.
5. DEWBERRY, E. B. Food poisoning. 3rd ed. Leonard Hill Ltd., London. 1950.
6. DOLMAN, C. E. Bacterial food poisoning. II. Staphylococcus food poisoning. Can. J. Public Health, 34 : 205-235. 1943.
7. DOLMAN, C. E. and WILSON, R. J. Experiments with staphylococcal enterotoxin. J. Immunol. 35 : 13-30. 1938.
8. DOLMAN, C. E. and WILSON, R. J. The kitten test for staphylococcus enterotoxin. Can. J. Public Health, 31 : 68-71. 1940.

9. DOLMAN, C. E., WILSON, R. J., and COCKROFT, W. H. A new method of detecting staphylococcus enterotoxin. Can. J. Public Health, 27 : 489-493. 1936.
10. EDDY, C. A. The frog test for staphylococcal enterotoxin. Proc. Soc. Exptl. Biol. Med. 78 : 131-134. 1951.
11. FULTON, F. Staphylococcal enterotoxin—with special reference to the kitten test. Brit. J. Exptl. Pathol. 24 : 65-72. 1943.
12. HAMMOND, W. McD. Staphylococcus enterotoxin: an improved cat test, chemical and immunological studies. Am. J. Public Health, 31 : 1191-1198. 1941.
13. HAYNES, W. C. and HUCKER, G. J. The food poisoning micrococci. Food Research, 11 : 281-297. 1946.
14. HUSSEMANN, D. L. and TANNER, F. W. A comparison of strains of staphylococci isolated from foods. Food Research, 14 : 91-97. 1949.
15. JONES, A. H. and LOCHHEAD, A. G. A study of micrococci surviving in frozen-pack vegetables and their enterotoxic properties. Food Research, 4 : 203-216. 1939.
16. NORTH, W. R. Staphylococcus enterotoxin in relation to alpha-hemolysin production in simple media. Food Research, 8 : 169-178. 1943.
17. RIGDON, R. H. Observations on Dolman's test for determining the presence of staphylococcal enterotoxin. Proc. Soc. Exptl. Biol. Med. 38 : 82-84. 1938.
18. ROBINTON, E. D. A rapid method for demonstrating the action of staphylococcus enterotoxin upon *Rana pipiens*. Yale J. Biol. and Med. 23 : 94-98. 1950.
19. SAINT-MARTIN, M., CHAREST, G., and DESRANLEAU, J. M. Bacteriophage typing in staphylococcal food poisoning. Can. J. Public Health, 42 : 351-358. 1951.
20. SHAUGHNESSY, H. J. and GRUBB, T. C. The incrimination of milk and milk products in staphylococcus poisonings. Can. J. Public Health, 28 : 229-234. 1937.
21. SINGER, A. and HAGAN, W. A. Staphylococcal toxins. J. Bacteriol. 41 : 74-75. 1941.
22. SLANETZ, L. W. Studies on staphylococcal toxins. J. Bacteriol. 43 : 105-106. 1942.
23. SURGALLA, M. J., BERGDOLL, M. S., and DACK, G. M. Some observations on the assay of staphylococcal enterotoxin by the monkey feeding test. J. Lab. Clin. Med. 41 : 782-788. 1953.
24. SURGALLA, M. J. and HITE, K. E. A study of enterotoxin and alpha and beta hemolysin production by certain staphylococcus cultures. J. Infectious Diseases, 76 : 78-82. 1945.
25. SURGALLA, M. J., KADAVY, J. L., BERGDOLL, M. S., and DACK, G. M. Staphylococcal enterotoxin: production methods. J. Infectious Diseases, 89 : 180-184. 1951.
26. VAN HEYNINGEN, W. E. Bacterial toxins. 1st ed. The Ryerson Press, Toronto, Ont. 1952.

STUDIES WITH STAPHYLOCOCCAL TOXINS

II. THE SPECIFICITY OF ENTEROTOXIN[1]

By F. S. Thatcher and B. H. Matheson

Abstract

The reported study of the known toxins of six different strains of enterotoxigenic staphylococci of diverse lysin productivity allows the conclusion that the enterotoxin, as indicated by the emetic response of cats, is a specific entity that is distinct from α-, β-, or δ-lysins. Emesis in cats may also be caused by β-lysin if injected in quantities greater than 80 units per kgm. The Dolman kitten test for enterotoxin may be made less equivocal by selective removal of all traces of lysins by boiling of the test specimen followed by incubation in the presence of ascorbic acid. Lysins inactivated by heat, formalin, homologous antitoxins, or ascorbic acid do not cause emesis. Immunization of cats with specific enterotoxic filtrates has demonstrated the existence of antigenically distinct enterotoxins.

Little is known of the nature of the emetic principle, the so-called enterotoxin that is produced by the strains of staphylococci able to cause food-poisoning (20, 23).

Fulton (9) concluded that the beta-lysin is responsible for the emetic reaction in cats which reaction may not be related to the occurrence of vomiting in man. With regard to the latter, Fulton argued that sufficient evidence is not available to show that the substance that functions as the enterotoxin in man is distinct from the alpha-lysin even though Dolman had previously shown that large quantities of alpha-toxin could be ingested by man with impunity (1). Dolman (5) and Surgalla (18) have observed vomiting in cats following injection of unheated preparations containing beta-lysin, but which were believed to be free from enterotoxin or alpha-lysin. Slanetz (17) has raised the possibility that the so-called enterotoxin may be the beta-lysin. Subsequent work by Dolman (2) seemed to negate this suggestion since cats were shown to react differently to the beta-lysin and enterotoxin while man did not react to comparatively large amounts of beta-lysin taken by the oral route. Singer and Hagan (16) state that the dermonecrotic toxin and the lethal toxin survived boiling for six hours. In the light of Levine's (11) evidence that the dermonecrotic and lethal toxins are identical with alpha-lysin, Singer's work would suggest that alpha-lysin may persist in preparations that have been boiled, ostensibly to destroy lysins, before testing for enterotoxin. Dolman and Wilson (4) and Fulton (9) refer to an anomalous heat-inactivation of the alpha-lysin, having found that this lysin is more inactivated at 57° C. than at temperatures above 80° C.

On the other hand, North (15) has shown that a number of strains that produced alpha-lysin were not enterotoxigenic, while Dolman (3) was able to inhibit the production of alpha-lysin by incubating cultures at room temperature without curtailing the enterotoxicity of a filtrate. Dolman and Wilson

[1] *Manuscript received January 26, 1955.*
A contribution from the laboratories of the Food and Drug Directorate Department of National Health and Welfare, Ottawa, Canada.

(4) and Dolman (2) also showed that inactivation of alpha-lysin in a strain of staphylococcus that lacked ability to produce beta-lysin did not destroy potentiality to cause emesis.

The conclusions of these latter investigators that no relationship exists between alpha- or beta-lysin and enterotoxin have been upheld by a number of other investigators whose work is discussed by Surgalla and Hite (19). Nevertheless, the recent text by Van Heyningen (23) draws attention to the continuing uncertainty concerning the specific roles of the various staphylococcal toxins in causing emesis.

A further possible cause for uncertainty arises from the observation of Marks and Vaughan (12) that delta-lysin is thermostable. Elek and Levy (7) have shown that, in general, coagulase-positive strains are consistent producers of alpha-, beta-, and delta-lysins, while Evans *et al.* (8) have stated that most enterotoxigenic strains of staphylococci produce the coagulase enzyme. Hence, until shown otherwise, the delta-lysin should be considered as a possible cause of the emesis induced by heated filtrates. Also, as already pointed out by the present authors (13), it has not been clearly shown whether or not an enterotoxic function may still persist in the molecules of specific lysins even though their lytic capacities may have been destroyed by heating. Moreover, the degree to which beta-lysin may contribute to an emetic reaction in cats needs further clarification.

It is the purpose of this paper to investigate the conflicting claims of the role of lysins in causing emetic reactions in the cat and to determine (1) whether a distinct entity exists in so-called enterotoxigenic strains which on intraperitoneal injection into cats causes vomiting in the absence of alpha-, beta-, and delta-lysins; (2) whether alpha-, beta-, or delta-lysins in boiled preparations of culture filtrates are able to cause characteristic vomiting in the absence of a specific enterotoxin; and if a specific lysin should be shown to be capable of inducing emesis, to determine (3) the minimal concentration of lysin necessary to provide a positive reaction from boiled preparations.

Methods

Young cats were accustomed to laboratory life and maintained, fed, and injected with test preparations by methods previously described by Matheson and Thatcher (13).

The criterion for enterotoxicity of specimens was the occurrence in the cat of multiple episodes of retching followed by decisive vomiting within a period of from 10 to 120 min. after intraperitoneal injection, and followed by complete recovery in less than 12 hr.

Specific lysins were detected by methods in accord with the scheme presented in Table I. The scheme is based upon a composite of information obtained from Topley and Wilson (22), Van Heyningen (23), and Marks and Vaughan (12) and upon the observation of Levine (11) of the unity of alpha-lysin, the dermonecrotoxin and the lethal toxin and the probability that all three

TABLE I

TESTS FOR THE PRESENCE OF SPECIFIC LYSINS IN FILTRATES OF *M. pyogenes* VAR. *aureus*

Source of erythrocytes (1% cells in final suspension)	Reaction	Lysin indication
Rabbit	Lysis at 37° C., 1 hr. No lysis after neutralization with alpha antitoxin*	Alpha
Sheep	Lysis at 37° C., 1 hr. Increment in lysis caused by 5° C., 1 hr., after 37° C., 1 hr. No increment at 5° C. 1 hr. neutralized with alpha antitoxin*; 37° C., 1 hr.; lysis after 5° C., 1 hr.	Alpha and/or delta Beta Alpha in excess of beta or beta absent Beta
Horse	Lysis at 37° C., 1 hr.	Delta
Man	Rapid lysis at room temperature	Delta plus beta
——	Dermonecrotoxin present (guinea pig, mice, rabbits)	Alpha

* *Alpha antitoxin obtained from commercial antitoxin after adsorption with a β-lytic filtrate free from α-lysin.*

responses are caused by the same toxic molecule. The lysin titers of staphylococcal preparations were estimated by the progressive dilution method using 1% red blood cells from the sheep, rabbit, horse, and man, each after washing three times with sterile physiological saline solution. Titers are expressed as the dilution of a specimen that causes hemolysis of 50% of the erythrocytes. The presence of a lethal toxin was noted by the occurrence of death of a test cat within 12–24 hr. after intraperitoneal injection. Aliquots of representative preparations that caused death in the cat were further tested for lethal toxin by intravenous injection of 0.1 ml. per kgm. into the lateral vein of the ear of a rabbit. Heated preparations and uninoculated media were similarly tested with a volume of 5 ml. per kgm.

Dermonecrotic action was determined by an intradermal injection using shaved rabbits, guinea pigs, or mice depending on the availability of the respective animal. The development of necrotic ulceration at the site of the injection was the criterion for the presence of this toxin.

The staphylococcus cultures used were strain L.16, which produced alpha-, beta-, and delta-lysins concurrently (Matheson and Thatcher (13)); strain 12069-alpha (4), which in Dolman's laboratory caused emesis in cats and produced alpha-lysin but no beta-lysin; strains 224 and 230 of Casman, which caused emesis and produced alpha-lysin at titers of 1 : 128 and 1 : 1 respectively, but no detectable quantities of beta- or delta-lysin; and strain J 32A, which is recorded as being non-enterotoxigenic but is a potent producer of beta-lysin (Surgalla (18)). All the former strains were initially implicated in episodes of food-poisoning.

The peptone–glucose medium of Dolman (6) was used for toxin production. Inoculated plates were incubated for 40 hr. at 37° C. in Brewer jars containing air with 30% carbon dioxide. The yield from 100 plates at a time was filtered first through four layers of cheese cloth and then through a Seitz "EK" filter pad to obtain sterility.

Test preparations included unheated culture filtrates, filtrates that had been boiled for 30 min., filtrates from uninoculated media, various preparations from culture filtrates containing concentrates of an emetic principle, and, in addition, specific fractions separated from the emetic factor during attempts at purification by methods to be described elsewhere (21).

Specific filtrates were neutralized by commercial staphylococcus antitoxin (Connaught Laboratories) or with beta-antitoxin prior to injection. As a further aid to the elucidation of the specificity of enterotoxin and in order to estimate the antigenic homogeneity of enterotoxin from different strains, three series each of five cats were respectively immunized against strains L.16, S.6, and 224. Immunization was accomplished by administering to each cat a series of nine injections at intervals of five days. The criterion for immunity to a specific strain was the failure of all cats in each series to react on two occasions to an injection of three times the final dose of the specific filtrate. Each series of cats were then injected, in turn, with specimens of filtrate from the other two strains. Cats immunized against L.16 were also injected with beta-lysin from strain J 32A.

As an aid to the clarification of the role of beta-lysin in relation to emesis in cats, a number of attempts were made to inactivate beta-lysin without destruction of enterotoxin. These included determinations of: (*a*) the comparative effect of temperatures of 57° C. and 100° C. for time intervals ranging from one minute to six hours, upon the inactivation of alpha- and beta-lysins [Fulton has reported that alpha-lysin is inactivated to a greater degree at the lower temperature (9)]; (*b*) the effect of formaldehyde upon the relative inactivation of alpha- and beta-lysins and upon enterotoxin [Dolman indicated that alpha-lysin is more readily inactivated by formaldehyde than is the enterotoxin (2)]; (*c*) the effect of ascorbic acid on the comparative rate of inactivation of the same three toxins [Mercier has stated that the hemolytic property of alpha-lysin may be inhibited by ascorbic acid (14)]; and (*d*) by a combination of boiling followed by the use of ascorbic acid.

Filtrates of strains L.16 and J 32A were used for the foregoing determinations. "Formalization" was accomplished by the addition of formaldehyde to filtrates to provide a concentration of 0.3% and incubation of the specimens at 37° C. for from one to seven days. Ascorbic acid was added to specific filtrates and to uninoculated media at rates ranging from 1 mgm. to 30 mgm. per ml. Specimens were shaken and allowed to stand for 18 hr. at 10° C., adjusted to pH 7.0, and dialyzed before determination of lysin titers and injection into cats.

A concentrate of delta-lysin was prepared by continuous washing of the Seitz filter pad used for separating the *Staphylococcus* cells from the culture

milieu with 30 ml. of absolute ethanol for 30 min. (Preliminary studies had shown that about half the delta-lysin content of the culture medium was retained on the filter pad.) The lysin was separated from the ethanol by dialysis and diluted to provide an aqueous injectant containing an amount of delta-lysin equivalent to that in from 10 to 15 ml. of unheated filtrate at a titer of 1 : 30.

Results

The Relationship of Lysins and Enterotoxin to Feline Emesis

The comparative lysin content of representative specimens of various heated and unheated filtrates of cultures of strain L.16 and specific fractions obtained therefrom and their individual ability to cause dermonecrotoxicity, lethality, and emesis in cats are listed in Table II.

TABLE II

TOXIN MANIFESTATIONS OF VARIOUS PREPARATIONS FROM FILTRATES OF *M. pyogenes* VAR. *aureus*, STRAIN L.16

	Toxin indication						
	Lysins (titer reciprocal)				Lethal titer	Enterotoxin	
Test preparation	Alpha	Beta	Delta	D.N.T.*	(No. of cats killed)	No. of cats tested	No. of cats positive
1. Culture filtrates L.16 (unheated)	512 (64–1024)	2048 (1024–2048)	2 (2–8)	+ +	10 10	10 10	10 10
2. Culture filtrate L.16 (heated 100° C., 30 min.)	0–4	1–8	0	0	0	200	197
3. Eluate from Seitz filter pad after filtration (L.16)	0	0	30	0	0	5	0
4. Heated, cooled, filtrate plus resuspended ppt. (L.16)	8	32	0	0	0	2	2
5. Ethanol ppt. from heated L.16 filtrates	0.02–0.2†	0.1–0.8†	0	0	0	75	73
6. Aq. insol. fraction of alc. ppt. (5)	0	0	0	0	0	2	0
7. Alc. sol'n after removal of ppt. (5) dialyzed, lyophilized	0	0	Trace	0	0	2	0

* *D.N.T. = dermonecrotoxin.*
† *Figure obtained by calculation from undiluted concentrate.*

The general range in lysin titers for different specimens of unheated filtrates of strain L.16 were, respectively, alpha 1 : 64 to 1 : 1024, beta 1 : 1024 to 1 : 2048 (one specimen developed a titer of 1 : 16,000), and delta 1 : 2 to 1 : 8. All specimens of unheated filtrates of strain L.16, without exception, caused emesis in and were fatal to cats, as well as being severely dermonecrotoxic to mice, guinea pigs, and rabbits. Lethal and dermonecrotoxic reactions from heated and unheated filtrates are reported in Table III. After 30-min.

TABLE III

LETHAL AND DERMONECROTOXIC REACTIONS IN CATS AND RODENTS TO CULTURE FILTRATES OF ENTEROTOXIGENIC STRAINS OF *M. pyogenes* VAR. *aureus*

Specimen	Lysin titers*			Lethal reaction		Dermonecrotoxic reaction		
	Alpha	Beta	Delta	Cats	Rabbits	Mice	Guinea pigs	Rabbits
L.16 unheated	512	1024	2	+	+	+	+	+
	1024	2048	8	+	+	+	+	+
	64	2048	4	+	+	+	+	+
L.16 boiled 30 min.	2	2	0	0	0	0	0	0
	4	8	0	0	0	0	0	0
	2	4	0	0	0	0	0	0
S.6 unheated	64	0	0	+	+	+	+	+
boiled 30 min.	0	0	0	0	0	0	0	0
224 unheated	128	0	0	+	+	+	+	+
boiled 30 min.	0	0	0	0	0	0	0	0

* *Reciprocals.*

boiling, the respective lysin titers in filtrates of strain L.16 were: alpha 0 to 1 : 4, beta 1 : 1 to 1 : 8, and delta 0. Of 200 tests cats receiving injections of these heated preparations, 197 vomited and showed characteristic ancillary reactions (13) but all test cats rapidly returned to normal health. Dermonecrotoxic reactions from heated filtrates were not encountered. Small amounts of beta-lysin were, however, consistently present in these heated filtrates with a maximum titer of 1 : 8. Those specimens that showed complete absence of alpha-lysin gave reactions identical to those containing the maximum residual amount of this lysin (titer 1 : 4). Table II also shows that the five cats receiving an injectant containing a concentrate of delta-lysin failed to vomit or to show any other sign of toxicity.

When culture filtrates were boiled and cooled a dense precipitate was formed. After four hours at 4° C., a further precipitate appeared. When these precipitates were resuspended in the filtrate by shaking, part of the original alpha- and beta-lysin activities were regained, the maximum titers recovered in this way being 1 : 8 and 1 : 32, respectively. Such specimens were neither lethal nor dermonecrotic, but caused vomiting.

Injectants prepared from a concentrate rich in an emetic principle that had been isolated from a culture filtrate by ethanol precipitation (21), when diluted with water to provide a concentration approximately equivalent to that present in the original filtrate, contained no detectable amounts of either alpha- or beta-lysins, though alpha- and beta-lysins were presumed to be present at the respective titers of 1 : 0.02 to 1 : 0.2 and 1 : 0.1 to 1 : 0.8 as calculated from the lytic reactions of the concentrates before dilution. Dermonecrotoxic and

TABLE IV

LYSIN CONTENT AND EMETIC REACTIONS FROM FIVE STRAINS OF *M. pyogenes* VAR. *aureus*—REPRESENTATIVE RESULTS

Specimen		Lysin titer (reciprocals)			Vol. injected per kgm. (ml.)	Time lapse after injection (min.)		No. of emetic spasms	Time of death (hr.)
		Alpha	Beta	Delta		First spasm	Last spasm		
L.16 filtrate									
Unheated	1	64	2048	2	1.0	7	75	10	24
	2	1024	2048	8	1.0	4	84	11	24
	3	512	1024	4	1.0	11	48	3	24
	4	512	4096	4	1.0	29	82	3	48
Boiled	1	2	2	0	0.5	27	87	5	Survived
	2	4	8	0	1.0	29	62	4	Survived
	3	2	4	0	1.0	41	92	4	Survived
	4	2	4	0	1.0	42	118	7	Survived
S.6 filtrate									
Unheated		64	0	0	1.0	42	54	2	24
Boiled	1	0	0	0	1.0	26	67	3	Survived
	2	0	0	0	1.0	43	80	2	Survived
	3	0	0	0	1.0	42	99	3	Survived
224 filtrate									
Unheated		128	0	0	1.0	14	83	4	24
Boiled	1	0	0	0	1.0	49	106	3	Survived
	2	0	0	0	1.0	47	79	3	Survived
	3	0	0	0	1.0	28	64	2	Survived
230 filtrate									
Unheated		1	0	0	1.0	37	69	3	Survived
Boiled		0	0	0	2.0	57	77	2	Survived
12069 α filtrate									
Unheated	1	512	0	2	0.5	—	70	4	24
	2	512	0	2	1.0	40	—	3	48
Boiled	1	0.2	0	0	1.0	42	123	3	Survived
	2	4	0	0	1.0	45	80	3	Survived
	3	1	0	0	1.0	45	95	4	Survived
Enterotoxin concentrates									
L.16*	1	0.02	0.1	0	0.5	27	87	5	Survived
	2	0.02	0.4	0	1.0	29	62	4	Survived
	3	0.02	0.4	0	1.0	42	118	7	Survived
S.6									
Unheated		?	0	0	1.0	49	72	3	24
Boiled		0	0	0	1.0	63	153	3	Survived
12069 α		0	0	0	0.2	37	64	4	Survived
Boiled		0	0	0	0.15	41	41	1	Survived

** Diluted to equivalence with 3× vol. of original filtrate.*

lethal activities were absent, but 73 of the 75 cats tested with such specimens gave positive emetic reactions. Those animals that failed to vomit had received volumes of injectant of less than 0.25 ml.

A concentrate prepared by Dr. Bergdoll from strain S.6 that produces no beta-lysin reacted similarly.

The data reported in Table IV show that these general findings apply to a number of other strains having widely divergent potentialities for lysin production. Table IV lists the lysin titers and the range in time between injection and inducement of emesis for five different enterotoxigenic strains.

Unheated filtrates from all five strains caused emesis in cats, and all except strains 230 were lethal to the test animals. The specimen of filtrate of strain 230 reported in Table IV contained no other lysin except alpha which occurred at the very low titer of 1 : 1. Table IV shows that emesis was also caused by filtrates that have been completely freed from lysins by boiling (strains S.6, 224, 230, and 12069 alpha).

The time of occurrence of the first emetic spasm is of the same order for all heated filtrates, which in turn, is similar to the time of emesis for unheated filtrates of strains that lack beta-lysin. Unheated specimens of filtrates from L.16 tended to cause vomiting after a shorter induction period when compared with filtrates of other strains or with heated preparations of the same strain. Filtrates of L.16 contain a relatively large amount of beta-lysin (titer 1 : 1024 to 1 : 4096). This suggests, in accord with other reports already cited, that beta-lysin may induce vomiting independently from enterotoxin.

In order to determine the minimal amounts of beta-lysin required to induce emesis, strain J 32A, which produces beta-lysin but is reported (2, 18) to be non-enterotoxigenic, was tested for its ability to cause vomiting in cats. Table V shows the effect of progressive dilution of both heated and unheated filtrates of J 32A upon emesis and lethality in cats.

Diluted, unheated filtrates of J 32A caused cats to vomit provided that beta-lysin was injected at the rate of not less than 80 units per kgm. No emetic reaction was noted when 66 units or less per kgm. were injected. No specimen containing 120 units or less caused death. All specimens containing 800 units or more proved fatal. The results expressed in Table V clearly show that the heated filtrate of strain J 32A behaved very differently from the unheated specimen when compared in terms of content of active lysin. When heated filtrates were used, vomiting occurred in the presence of 16 units or more of beta-lysin, while one of seven cats vomited after receiving eight units. Since a few specimens of heated filtrates of L.16 contained beta-lysin at a titer of 1 : 8 the possibility was entertained that emetic reactions might on occasion be caused by this low level of beta-lysin and hence invalidate conclusions concerning the presence of enterotoxin.

Table VI expresses the beta-lysin content for both unheated and boiled preparations of the specimens of J 32A containing, respectively, the lowest amount of lysin that consistently caused emesis and the highest amount that failed to cause emesis in terms of the equivalent volume of the original filtrate.

TABLE V

THE EFFECT ON CATS OF β-LYSIN FROM A β-HEMOLYTIC STRAIN* OF *M. pyogenes* VAR. *aureus* (STRAIN J 32A)

Amount of β-lysin injected (units per kgm.)	Time of emesis		No. of emetic spasms	No. of diarrhea emissions	Time of death (hr.)
	1st spasm	Last spasm			
Unheated filtrates					
1000	11	21	2	5	48
1000	8	67	7	4	24
1000	7	44	6	6	24
800	—	—	—	7	24
120	13	27	3	2	—
120	11	32	3	3	—
85	19	41	3	4	—
80	25	32	2	2	—
66	—	—	—	—	—
64—12 (18 cats)	—	—	—	—	—
Heated filtrates					
128	13	32	3	3	24
128	9	23	3	2	—
68	16	32	2	1	12
68	14	14	1	0	—
67	27	38	2	1	—
38	18	29	3	3	36
36	28	43	2	3	36
32	15	49	2	1	72
18	40	40	1	—	—
16	32	32	1	2	—
16	42	42	1	1	—
12	—	—	—	—	—
8	43	43	1 (1 cat)	—	—
8	—	—	6 (6 cats)	—	—

* *Reported to be non-enterotoxigenic.*

TABLE VI

THE ASSOCIATION BETWEEN BETA-LYSIN AND AN EMETIC REACTION IN CATS (STRAIN J 32A) *M. pyogenes* VAR. *aureus*)

Emetic preparations		Non-emetic preparations	
Smallest amount of lysin in filtrate causing emesis (units/kgm.)	Equivalent volume of filtrate (ml./cat)	Greatest amount of lysin in filtrate not causing emesis (units/kgm.)	Equivalent volume of filtrate (ml./cat)
Unheated filtrates			
80	0.06	66	0.05
Boiled filtrates			
16	1.0		
8*	0.8	8	0.5–0.7

* *One of seven cats reacted to this amount.*

Unheated specimens diluted to provide an injectant containing 80 units of beta-lysin, the lowest amount to cause emesis from an unheated specimen, contained only 0.08 ml. of the original filtrate, but the 32 units in a heated specimen that caused emesis (provided by an injection of 2 ml. at a beta-titer of 1 : 16) were obtained from 0.8 ml. of filtrate. This result suggests that heated filtrates of our preparations from J 32A contained an emesis-inducing factor other than active beta-lysin. Results of an attempt to verify this observation by injection of heated J 32A filtrates after "neutralization" of beta-lysin with a commercial staphylococcus antitoxin containing anti alpha- and beta-lysin activities are shown in Table VII. The data in this table shows that after removal of all beta-lysin activity from a filtrate of strain J 32A a capacity to cause emesis remains, though to a lesser degree than in the unheated filtrate. Control preparations of antitoxin alone and with uninoculated media caused no emesis. The same effect was shown by use of the antitoxin preparation with strains S.6, 224, and L.16. Filtrates of S.6 and 224 contain no beta-lysin. This suggests that the antitoxin used contained some degree of antienterotoxin activity. Enquiry of the manufacturers revealed that the antitoxin had, indeed, been prepared from a group of cultures that included enterotoxigenic strains. Subsequently, the use of an antitoxin prepared from purified beta-lysin removed all beta-lysin activity from filtrates of strain J 32A but did not destroy its emetic capacity. Hence, further experiment became necessary to determine the true cause of an emetic reaction that might arise from an injectant containing eight units of beta-lysin, such as had been encountered with strain L.16.

Table VIII summarizes the results of an experiment designed to establish more clearly the potential emetic capacity of beta-lysin alone. Heated filtrates of strains L.16, S.6, and 224 were used, respectively, to immunize three groups of five cats. (Filtrates of S.6 and 224 contain no beta-lysin.) The data in Table VIII shows that after the immunizing process all cats failed to react to an injection of the homologous filtrate. When, however, specimens of filtrates of each of the other two strains were injected into the immune animals, immunization against a specific strain failed to confer immunity to each of the other strains (Table IX). Partial immunity to strain 224 was provided by immunization to strain L.16, but this occurred in no other combination of strains.

When preparations of filtrates of J 32A were heated to contain an amount of beta-lysin equivalent to the residual lysin in heated specimens of L.16 and injected into cats immunized against L.16, four of the five cats vomited. This again points to the probability that our filtrates of J 32A contained a substance other than beta-lysin that could cause cats to vomit, and also shows that the heat-inactivated lysin molecule was not this substance since L.16 filtrates contained a greater amount of heat-inactivated lysin than did filtrates of J 32A.

TABLE VII

THE EFFECT OF ANTITOXIN* (COMMERCIAL) ON EMESIS, DIARRHEA, AND DEATH IN CATS CAUSED BY HEATED FILTRATES OF VARIOUS STRAINS OF *M. pyogenes* VAR. *aureus*

		Heated filtrates				Heated filtrates plus antitoxin			
	Vol. injected (ml.)	β-lysin content (units†)	Emesis (no. of spasms)	Diarrhea (no. of emissions)	Death (time of occurrence, hr.)	β-lysin content (units†)	Emesis (no. of spasms)	Diarrhea (no. of emissions)	Death (time of occurrence, hr.)
Strain									
J 32A	5	60	6	3	24	0	1	0	Survived
	2	24	2	2	Survived	0	0	1	Survived
S.6	5	0	4	3	Survived	0	1	0	Survived
	2	0	2	2	Survived	0	0	1	Survived
224	5	0	1	3	Survived	0	0	0	Survived
	2	0	1	2	Survived	0	0	1	Survived
L.16	5	20	3	2	Survived	0	0	0	Survived
	5	40	4	1	Survived	0	0	0	Survived
	5	40	2	2	Survived	0	0	0	Survived
	2	8	2	1	Survived	0	0	0	Survived
	2	16	3	2	Survived	0	0	0	Survived
	2	16	2	3	Survived	0	0	0	Survived
Control preparations									
Antitoxin (8%) + saline	5 (3 cats)	0	0	0	Survived				
	2 (3 cats)	0	0	0	Survived				
Uninoculated media	5 (3 cats)	0	0	0	Survived				
	2 (3 cats)	0	0	0	Survived				

* *Antitoxin added to make 4% of volume of injectant.*
† *Units = reciprocal of titer × no. of ml. injected.*

TABLE VIII

EMESIS AND DIARRHEA IN CATS SUBJECTED TO IMMUNIZATION TO ENTEROTOXIGENIC FILTRATES OF DIFFERENT STRAINS OF *M. pyogenes* VAR. *aureus*

Strain	Cat No.	Reactions to injections at progressive time intervals																	
		1st injection		2nd (5 days)		3rd (10 days)		4th (15 days)		5th (20 days)		6th (25 days)		7th (30 days)		8th (35 days)		9th (40 days)	
		E.*	D.†	E.	D.	E.	D.	E.	D.	E.	D.	E.	D.	E.	D.	E.	D.	E.	D.
L.16	151	4	0	3	1	3	1	0	0	0	0	0	1	0	0	0	0	0	0
	152	4	1	5	2	1	0	0	0	1	1	0	0	0	0	0	0	0	0
	153	4	2	3	2	3	0	2	1	0	0	0	0	0	2	0	0	0	0
	154	3	1	3	2	3	3	2	1	0	0	1	0	0	1	0	0	0	0
	155	7	1	6	1	6	2	3	1	0	1	1	3	1	0	0	0	0	0
S.6	156	1	0	2	0	1	1	0	1	0	1	0	1	0	0	0	0	0	0
	157	2	1	2	0	3	1	0	1	0	0	0	2	0	0	0	0	0	0
	158	3	2	2	1	1	0	1	0	1	1	1	2	0	0	0	0	0	0
	159	3	1	2	2	2	3	1	1	2	0	0	0	1	1	0	0	0	0
	160	2	1	3	1	2	2	2	1	1	1	0	3	1	0	0	0	0	0
224	161	3	2	2	2	1	0	2	1	0	0	1	0	0	2	0	0	0	0
	162	2	0	2	1	2	1	1	0	0	0	1	1	0	1	0	0	0	0
	163	2	1	2	1	0	0	0	0	0	0	0	0	0	0	—	—	—	—
	164	2	6	1	3	1	2	0	0	0	0	0	1	0	1	0	0	0	0
	165	3	4	2	3	2	0	1	1	0	1	0	1	0	0	0	0	0	0

* *Emesis* (*no. spasms*).
† *Diarrhea* (*no. emissions*).

TABLE IX

ANTIGENIC DIFFERENCES AMONG ENTEROTOXINS FROM DIFFERENT STRAINS OF *M. pyogenes* VAR. *aureus*

Filtrate injected (boiled 30 min.)	Filtrate to which immunized					
	L.16		S.6		224	
	Emetic reactions		Emetic reactions		Emetic reactions	
	Positive	Negative	Positive	Negative	Positive	Negative
L.16	0	5	5	0	5	0
S.6	4	1	0	5	5	0
224	1	4	4	1	0	5
J 32A	4	1	—	—	—	—

The Selective Removal of Lysins from Enterotoxic Filtrates

The results of studies made to remove alpha- and beta-lysins from a filtrate without destruction of enterotoxin are summarized in Tables X, XI, and XII.

TABLE X

THE EFFECT OF DIFFERENTIAL TEMPERATURES ON THE SURVIVAL OF LYSINS FROM DIFFERENT STRAINS OF *M. pyogenes* VAR. *aureus*

Test strain	Lysin titers after heating								
	Unheated			57° C. 30 min.			100° C. 30 min.		
	Alpha	Beta	Delta	Alpha	Beta	Delta	Alpha	Beta	Delta
L.16	1024	2048	4	4	16	0	8	4	0
224	128	0	0	4	0	0	8	0	0
230	4	0	0	0	0	0	0	0	0
S.6	256	0	1	0	0	0	4	0	0
12069-alpha	512	0	1	2	0	0	8	0	0

TABLE XI

THE RATE OF INACTIVATION OF BETA-LYSIN DURING BOILING AND THE SURVIVAL OF LYSIN IN THE RESULTANT PRECIPITATE (STRAIN J 32A, *M. pyogenes* VAR. *aureus*)

Duration of boiling (min.)	Centrifuged filtrate		Precipitate resuspended in filtrate	
	Lysin titer (reciprocal)	Loss of activity (%)	Lysin titer	Loss of activity (%)
0	1024	0	1024	0
1	256	75	256	75
3	128	87.5	256	75
5	128	87.5	128	87.5
7	128	87.5	128	87.5
10	64	93.8	128	87.5
15	32	96.8	64	93.8
20	32	96.8	32	96.8
25	16	98.4	32	96.8
30	16	98.4	32	96.8
35	16	98.4	32	96.8
40	8	99.2	16	98.4
50	8	99.2	16	98.4
60	4	99.6	8	99.2

TABLE XII

THE EFFECT OF FORMALDEHYDE ON THE ACTIVITY OF β-LYSIN AND ON THE EMETIC REACTION IN CATS (STRAIN L.16, UNHEATED; 0.3% FORMALDEHYDE, 37° C.)

Specimen	Duration of exposure to CH_2O (days)	β-Lysin titer	Emesis in cats
Control filtrate	0	1 : 2048	+
Filtrate + 0.3% CH_2O	2	1 : 64	+
Filtrate + 0.3% CH_2O	3	1 : 16	+
Filtrate + 0.3% CH_2O	4	1 : 8	0
Filtrate + 0.3% CH_2O	5	1 : 8	0
Filtrate + 0.3% CH_2O	6	1 : 4	0
Filtrate + 0.3% CH_2O	7	1 : 1	0

The rate of inactivation of alpha-lysin is shown to be slightly less at 100° C. than at 57° C. (Table X). After an exposure of 30 min. to the respective temperatures, unheated filtrates from two strains had a residuum of four units of alpha-lysin at 57° C. and eight units at 100° C. One filtrate (S.6) with an initial titer of 1 : 256 was completely freed from alpha-lysin at 57° C., but had a residual titer of 1 : 4 after boiling. No such anomaly with regard to relative rate of inactivation at the two temperatures was noted for beta-lysin. The higher temperature inactivated the beta-lysin the more rapidly. The precipitate caused by heating a toxic filtrate was shown to possess some beta-lysin activity (Table XI). After exposure to 100° C. for three minutes the loss of beta-lysin activity from the supernatant after centrifuging was 87.5%; after resuspension of the filtrate only a 75% loss was apparent. After 30-min. heating, the respective percentage losses in activity were 98.4 and 96.8. With an initial titer of 1 : 1024 this difference would amount to 16 lysin units per milliliter. After 60 min., the comparative reductions in beta-lysin content for the two preparations were, respectively, 99.6% and 99.2%.

Table XII shows the effect of incubating a filtrate of strain L.16 in the presence of 0.3% formaldehyde upon the survival of beta-lysin and of enterotoxin. The data show that it was not possible by this means to remove all beta-lysin from a filtrate having an initial titer of 1 : 2048 without also destroying all detectable enterotoxin. After three days of incubation, emetic reactions were not obtained. At this time the beta-lysin titer remained at 1 : 16.

Table XIII reports the data from a study of the effect of the addition of progressive amounts of ascorbic acid upon the activities of both alpha- and beta-lysins and upon enterotoxin. Addition of ascorbic acid to an uninoculated medium is shown to be without effect upon the normal non-emetic activity of the medium until an amount of ascorbic acid is added to provide an injectant with between 112 and 180 mgm. of acid per kgm. Beyond this amount, a single ejection of vomitus was induced in each test cat. When added to an unheated filtrate containing alpha- and beta-lysins at titers of

TABLE XIII

THE EFFECT OF ASCORBIC ACID ON LYSINS, DERMONECROSIS, LETHALITY, AND EMESIS OF UNHEATED FILTRATES OF *M. pyogenes* VAR. *aureus* STRAIN L.16

Specimen	Lysin titers		Dermonecrosis (Guinea pigs)	Lethality (Rabbits)	Emesis (Cats)
	α	β			
1. Unheated filtrate	164	2048	+	+	+*
2. Unheated filtrate plus ascorbic acid (30 mgm./cc.)	0	0	−	−	+
3. Specimen 2 after dialysis	8	0	−	−	+
4. Unheated filtrate followed by separate injection of ascorbic acid as in (2)	164	2048	+	+	+*

* *Death within 12 hr.*

1 : 1024 and 1 : 2048, respectively, no trace of alpha- or beta-lysin was detectable in the filtrate in the presence of quantities of ascorbic acid that provided an injectant of 17 mgm. or more per kgm. Removal of the acid from a filtrate by dialysis allowed the recovery of alpha-lysin activity to a titer of 1 : 8 but had no demonstrable effect upon the inactivated beta-lysin. Characteristic vomiting is induced by enterotoxigenic filtrates in the presence of up to 130 mgm. per kgm. of ascorbic acid, which was the highest concentration tested. The dermonecrotoxic and lethal properties of the filtrate were also destroyed by addition of ascorbic acid, using rabbits and guinea pigs to detect the former property, and cats and rabbits the latter (Table XIII).

To avoid possible adverse effects of high acidity (ascorbic acid added to a culture filtrate at rates of 12 and 30 mgm. per milliliter respectively, induce pH values of 4.4 and 3.9), lower amounts of ascorbic acid were added to a filtrate after removal of the greater part of the initial lysin content by heating. The data reported in Table XIV show that filtrates boiled for 15 min. and having residual lysin titers of 1 : 2 and 1 : 16 for alpha- and beta-lysins, respectively, were rendered completely free from either lysin by treatment with ascorbic acid at the rate of 5 mgm. per milliliter of filtrate. This treatment provided filtrates that evoked characteristic emetic reactions in all test animals. The resultant pH of 5.2 had previously been shown by the use of buffers to have no inactivating effect on "purified" enterotoxin concentrate.

Discussion

The foregoing results clearly show that culture filtrates of enterotoxigenic strains of *M. pyogenes* var. *aureus* cause emesis in cats because of the presence of a specific entity that is distinct from alpha-, beta-, or delta-lysins. This is evident from the following facts: (1) with consistent negative effect of control preparations, emesis resulted from strains that lacked alpha-, beta-, and delta-lysins or any combination of these; (2) emesis was induced by strains that

TABLE XIV

THE EFFECT OF ASCORBIC ACID* UPON THE ACTIVITIES OF α- AND β-LYSINS AND UPON EMESIS IN CATS IN HEATED AND UNHEATED FILTRATES OF *M. pyogenes* VAR. *aureus* (STRAIN L.16)

Ascorbic acid plus control medium			Ascorbic acid plus unheated filtrate (L.16)					Ascorbic acid plus boiled filtrate (L.16)					
Acid content of injectant (mgm./kgm.)	Emesis (no. of spasms)	Diarrhea (no. of emissions)	Acid content of injectant (mgm./kgm.)	Lysin titers α	Lysin titers β	Emesis (no. of spasms)	Diarrhea (no. of emissions)	Acid content of injectant (mgm./kgm.)	Lysin titers α	Lysin titers β	Emesis (no. of spasms)	Diarrhea (no. of emissions)	Resultant pH
0	0	0	0	1 : 1024	1 : 2048	4	5	0	1 : 2	1 : 16	3	2	7.5
5	0	0	17	0	0	2	3	1	0	1 : 16	3	4	6.9
10	0	0	19	0	0	1	7	5	0	0	4	2	5.2
12	0	0	86	0	0	2	3	8	0	0	3	1	4.8
18	0	0	130	0	0	4	3	12	0	0	3	2	4.4
22	0	0						20	0	0	2	3	4.1
86	0	0						30	0	0	3	3	3.9
112	0	0											
180	1	1											
210	1	1											
225	1	0											

* *Added to filtrates and kept at 10° C. for 18 hr. before test.*

produce alpha- and beta-lysins after removal of these lysins whether by heat, neutralization with antilytic antitoxins, or by the addition of ascorbic acid to lytic filtrates; (3) an emetic concentrate prepared from lytic strains but which contained no detectable lysins consistently caused emesis; (4) a concentrate of delta-lysin failed to cause emesis.

Beta-lysin from unheated filtrates may also cause emesis provided it is injected in quantities greater than 80 units per kgm. These amounts did not occur in any of our specimens of boiled filtrates. It is possible that smaller amounts from heated filtrates (16 units/kgm. or greater) may cause emesis in the presence of subemetic amounts of enterotoxin.

The heat-inactivated lysin would not seem to be the cause of emesis at normal levels of injection of heated filtrates as shown by inducement of emesis by a specific heated filtrate in animals immunized to a preparation containing greater amounts of heated lysin than in the specific injectant.

Our experiments revealed no evidence that alpha-lysin survives boiling to a degree to cause confusion with any other emetic principle, but showed that relatively large amounts of beta-lysin may behave similarly to the alpha-lysin to the extent of causing death.

It would appear that different strains of staphylococci possess enterotoxins of heterologous antigenicity as shown by the failure of immunization to a specific strain to confer immunity to other strains. This might well provide partial explanation of the reported low degree of antigenic capacity and lack of therapeutic value of non-autogenous antisera (10), though confirming Dolman's demonstration of positive antigenicity of enterotoxin (3).

It is possible that the conclusion with regard to heterologous antigenicity may not apply to the two strains L.16 and 224 since the enterotoxic capacity of the latter is less than that of L.16. Thus it must be considered that strain 224 may have failed to confer immunity to L.16 because the immunizing dose of 224 was quantitatively less than the "challenge" dose of L.16. No reason to suspect such a relationship between the other strains was noted.

The combined use of heat and ascorbic acid for complete inactivation of lysins may render the cat test for enterotoxin even less subject to equivocation. It is possible that some confusion may have arisen in the past by failure to remove precipitates from a boiled filtrate since the precipitate may contain active beta-lysin. Our observation that ascorbic acid destroys the lethal and dermonecrotic properties of a toxic filtrate as well as the alpha-hemolysin is not in accord with that of Mercier (14) who reported a selective action against hemolysis alone. Mercier neutralized his preparation after incubating it for one hour. We neutralized ours after incubating it for 18 hr. In addition to inactivation of alpha-lysin our method of using ascorbic acid also destroyed the hemolytic and lethal properties of the beta-lysin.

The indication of the presence of a small amount of an emetic substance in strain J 32A suggests to us that the cat test is highly sensitive and may reveal amounts of toxin that would be inactive *per os* in cats, monkeys, or humans. Alternatively, as pointed out personally by Dolman, even though the

demonstration of lysis was inhibited by beta-antitoxin, it is theoretically possible that other toxic properties of the beta-lysin may not have been neutralized. Moreover, it is possible that strain J 32A, after having been subjected to frequent transfer, may have developed an ability to produce enterotoxin under the conditions of culture used in these experiments which were designed to favor production of enterotoxin.

It is of some interest to note that a concentrate of an enterotoxic principle prepared by Dr. M. S. Bergdoll at the University of Chicago and which was known to cause emesis in monkeys, after it was heated to remove its content of alpha-lysin, behaved identically in cats as did our own cat-reactive concentrates and heated filtrates.

A final conclusion the authors would like to offer is that the pertinent results presented in this paper endorse the validity of the "Dolman kitten-test" and substantiate the deductions of Dolman and co-workers that enterotoxin is a specific substance distinct from known lysins.

Acknowledgment

The authors would like to acknowledge a number of helpful suggestions incorporated in this paper in response to a critique of an earlier manuscript kindly offered by Professor C. E. Dolman, of the University of British Columbia.

References

1. DOLMAN, C. E. Ingestion of staphylococcus exotoxin by human volunteers. J. Infectious Diseases, 55 : 172-183. 1934.
2. DOLMAN, C. E. Bacterial food-poisoning. II. Staphylococcus food-poisoning. Can. J. Public Health, 34 : 205-235. 1943.
3. DOLMAN, C. E. Antigenic properties of staphylococcus enterotoxin. Can. J. Public Health, 35 : 337-351. 1944.
4. DOLMAN, C. E. and WILSON, R. J. Experiments with staphylococcal enterotoxin. J. Immunol. 35 : 13-31. 1938.
5. DOLMAN, C. E. and WILSON, R. J. The Kitten-test for staphylococcus enterotoxin. Can. J. Public Health, 31 : 68-71. 1940.
6. DOLMAN, C. E., WILSON, R. J., and COCKCROFT, W. H. A new method of detecting staphylococcus enterotoxin. Can. J. Public Health, 27 : 489-493. 1936.
7. ELEK, S. D. and LEVY, E. Distribution of hemolysins in pathogenic and non-pathogenic staphylococci. J. Pathol. Bacteriol. 62 : 541-554. 1950.
8. EVANS, J. B., BUETTNER, L. G., and NIVEN, C. F. Evaluation of the coagulase test in the study associated with food-poisoning. J. Bacteriol. 60 : 481-484. 1950.
9. FULTON, F. Staphylococcal enterotoxin—with special reference to the kitten-test. Brit. J. Exptl. Pathol. 24 : 65-72. 1943.
10. HAYNES, W. C. and HUCKER, G. J. A review of micrococcus enterotoxin food-poisoning. Food Research, II : 281-297. 1946.
11. LEVINE, B. S. Unity of haemolytic, dermonecrotic and lethal properties of staphylococcal exotoxin and their corresponding counterparts in staphylococcal antitoxin J. Pathol. Bacteriol. 48 : 291-298. 1939.
12. MARKS, J. and VAUGHAN, A. C. T. Staphylococcal delta-hemolysin. J. Pathol. Bacteriol. 62 : 597-615. 1950.
13. MATHESON, B. H. and THATCHER, F. S. Studies with staphylococcal toxins. I. A reappraisal of the validity of the "kitten-test" as an indication of staphylococcal enterotoxin. Can. J. Microbiol. 1 : 372-381. 1955.
14. MERCIER, P. Sur le mechanisme de l'inhibition du pouvoir hemolytique. Compt. rend. soc. biol 127 : 297-299. 1938.

15. NORTH, W. R. Staphylococcus enterotoxin in relation to alpha-hemolysin production in simple media. Food Research, 8 : 169-178. 1943.

16. SINGER, A. and HAGAN, W. A. Staphylococcal toxins. J. Bacteriol. 41 : 74-75. 1941.

17. SLANETZ, L. W. Studies on staphylococcal toxins. J. Bacteriol. 43 : 105-106. 1942.

18. SURGALLA, M. J. A study of the production of staphylococcal enterotoxin in chemically defined mediums. J. Infectious Diseases, 81 : 97-111. 1947.

19. SURGALLA, M. J. and HITE, K. E. A study of enterotoxin and alpha- and beta-hemolysin production by certain staphylococcus cultures. J. Infectious Diseases, 76 : 78-82. 1945.

20. SURGALLA, M. J., KADAVY, J. L., BERGDOLL, M. S., and DACK, G. M. Staphylococcal entertoxin—production methods. J. Infectious Diseases, 89 : 180-184. 1951.

21. THATCHER, F. S., MATHESON, B. H., and SIMON, W. R. Studies with staphylococcal toxins. III. The application of paper electrophoresis to the resolution of components of toxic concentrates. Can. J. Microbiol. 1 : 401-411. 1955.

22. TOPLEY and WILSON'S. Principles of bacteriology and immunity. 3rd ed. *Revised by* G. S. Wilson and A. A. Miles. Edward Arnold & Co., London. 1946. pp. 1-970.

23. VAN HEYNINGEN, W. E. Bacterial toxins. 1st ed. The Ryerson Press, Toronto, Ont. 1950.

STUDIES WITH STAPHYLOCOCCAL TOXINS

III. THE APPLICATION OF PAPER IONOPHORESIS TO THE RESOLUTION OF COMPONENTS OF TOXIC CONCENTRATES[1]

By F. S. Thatcher, B. H. Matheson, and W. R. Simon

Abstract

A substance present in culture filtrates of enterotoxigenic staphylococci that is distinct from alpha-, beta-, or delta-lysins causes vomiting in cats when injected by the intraperitoneal route. An emetic fraction containing only vestigial traces of lysins can be resolved into three protein components and one polysaccharide by use of the Durrum ionophoresis method. The only component of this group that gave indications of enterotoxicity was a protein with specific mobility relative to the others. Hemolytic proteins were separated by paper ionophoresis using the method of McKinley, Maw, Oliver, and Common.

Dack and associates (1, 2, 7) at the University of Chicago have prepared concentrates of the staphylococcal enterotoxin by methods based upon use of the precipitants saturated ammonium sulphate solution, phosphoric acid at pH 3.5, and ethanol; or by chromatographic adsorption by specific resins such as "Hyflo-supercel" (Johns-Manville).

These same principles have been applied in the present study in order to concentrate and partially purify the substance produced by enterotoxigenic strains of staphylococci that causes emesis when injected into cats by the intraperitoneal route. The difference between criteria for enterotoxin as used in this work and that of the Chicago group should be noted. The latter prefer oral administration to monkeys rather than intraperitoneal injection of cats. Proof is not yet available that the two responses are caused by an identical substance.

Precipitation with ethanol provided a more consistent "yield" of enterotoxin so that our efforts towards fractionation have been largely directed towards refinement of the ethanol precipitation procedure. In addition, with the advent of paper-ionophoresis as a means of separating the protein components of a complex solution (3, 4), attempts have been made to use this new tool in order to provide further purification of the enterotoxin concentrates.

Methods

Enterotoxic filtrates were prepared and tests for an emetic substance and for specific lysins were carried out by the methods described by Thatcher and Matheson (8). The test organisms used were two specific strains, L.16 and S.6, of *Micrococcus pyogenes* var. *aureus*. Strain L.16 had previously been shown to produce alpha, beta, and delta-lysins as well as a substance that induced the emetic reaction in cats. Strain S.6 produced alpha-lysin and a

[1] *Manuscript received January 26, 1955.*

A contribution from the laboratories of the Food and Drug Directorate of the Department of National Health and Welfare, Ottawa, Canada.

small amount of delta-lysin, and, in addition, its culture filtrate has been shown to cause the emetic reaction in cats by the intraperitoneal route (8) and in monkeys when administered with a stomach tube (7).

Precipitation of Enterotoxin with Ammonium Sulphate

With the use of saturated ammonium sulphate for precipitation of the enterotoxin the following procedure was adopted: A culture was Seitz-filtered, boiled for 30 min., cooled to 4° C., and held for five hours; the resultant precipitates were removed by centrifuging and ammonium sulphate was added to saturation to the remaining solution which was then held at 5° C. for 24 hr. The precipitate so formed was removed by filtration, placed in a dialyzing sac, and suspended in running water at 10° C. for about 12 hr. or until such time as no indication of residual sulphate within the sac could be obtained by testing with barium chloride. The contents of the sac were centrifuged to remove any undissolved precipitate and the resultant solution diluted to a desired degree of equivalence to the original filtrate, or lyophilized for later reconstitution prior to injection. The filtrate remaining after the removal of the precipitate induced by ammonium sulphate was also dialyzed, lyophilized, and reconstituted in water prior to testing in cats.

Precipitation of Enterotoxin with Phosphoric Acid

For acid precipitation, the boiled and cooled filtrate was adjusted to pH 3.5 with phosphoric acid, held at 5° C. for 24 hr., centrifuged at 2000 r.p.m. for 10 min., and, after decanting, the precipitate was dialyzed and reconstituted or lyophilized as in the ammonium sulphate sequence. The supernatant solution was similarly dialyzed before being tested in cats.

Precipitation of Enterotoxin with Ethanol

After preliminary experiments to determine optimum temperatures using a range from −20° C. to +10° C., precipitation by ethanol was carried out by adding cooled absolute ethanol (−10° C.) to the boiled, cooled (5° C.), and filtered culture-filtrate until the filtrate contained 40% ethanol which was then held at from −15° C. to −20° C. for 24 hr. The specimen was centrifuged in precooled centrifuge vessels at 2000 r.p.m. for three minutes with precaution being taken to prevent the temperature of the alcoholic solution from rising above −10° C. during the process. The precipitate was recovered by decanting, dialyzed in water at 4° C., and the aqueously insoluble part of the precipitate removed. The resultant solution was subjected to the alcohol precipitation repeatedly (usually three times) until all of the alcohol-induced precipitate would dissolve almost immediately in a small volume (1–5 ml.) of water. The final clear, aqueous solution was dialyzed and aliquots from this specimen and from fractions at all stages of the procedure were tested for enterotoxin and for specific lysins.

Chromatographic Separation of Enterotoxin

An emetic preparation was also obtained by the use of an adsorption column of the resin "Hyflo-supercel". Boiled filtrates were adjusted at pH 6.35 with a citrate–phosphate buffer, before passing through the column, which was then washed with buffer at the same pH value, and elution accomplished with buffer at pH 7.8 (2). The eluate was dialyzed in water before use.

Fractionation of Toxic Preparations by Paper Ionophoresis

Concentrates of specific culture filtrates obtained by direct lyophilization and the aqueously soluble fraction of the ethanol-induced precipitate prepared as described above were subjected to paper ionophoresis in attempts to separate active toxic components of the respective specimens.

Initially, ionophoresis equipment was used in which the paper was held in a horizontal plane in accord with the modification by McKinley *et al.* (5) of the method of Cremer and Tiselius (3). For these particular experiments the test specimen of a lyophilized unheated filtrate was prepared from a culture filtrate from which possibly confusing material of large molecular size present in the medium had been removed by dialysis before inoculation. A series of drops of concentrate were placed across the center of a strip of filter paper previously saturated with buffer ("Veronal"—pH 8.4) and exposed for progressive times to an accurately controlled electrical current. With a veronal buffer at pH 8.4, an e.m.f. of 200 v. caused a current flow of 12 ma. After specific time intervals a narrow strip was cut from the filter paper and tested for the presence of protein with a solution of bromphenol blue (3). After establishing the position on the paper of various components of the original solution, the respective corresponding areas in the paper were cut out and eluted in saline prior to testing for lysins and enterotoxin. If enterotoxin were separated in this way the amounts obtained were either too small to cause emesis or were adsorbed upon the filter paper. Accordingly, the method of Durrum (4), using the continuous flow technique made possible by use of the paper in a vertical plane, was applied in order to allow accumulation of larger amounts of specific fractions. With this equipment the movement of proteins through the buffered filter paper could be traced by examining the paper in a darkened room while exposed to a beam of ultraviolet light. A greenish fluorescence indicated the presence of the proteins, this fact being later confirmed by use of the dyes bromphenol blue and naphthalene-black 12B, and by a positive ninhydrin reaction in aliquots of the pertinent fractions. Tests for polysaccharide were carried out using the anthrone reagent, on the assumption that a carbohydrate that was non-dialyzable was a polysaccharide. The applied voltage could be modified to provide, within limits, the desired degree of separation after having observed the fluorescent pathways. After preliminary trials the whole apparatus exclusive of the voltage control was kept in a refrigerator at 8° C. ± 2° C.

The effect upon the comparative degree of separation of the component proteins of emetic concentrates was determined using a variety of buffers at different ionic strengths and with a broad range of current flow.

The buffers used included "Veronal" (diethyl-barbituric acid and sodium diethylbarbiturate) at pH values ranging from 7.5 to 9.6 and at ionic strengths ranging from 0.1 to 0.01; and the various buffers recommended for use in electrophoresis by Miller and Golder (6) at pH values, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, each at an ionic strength ranging from 0.1 to 0.001. A solution of acetic acid at pH 4.1 and ionic strength 0.0005 was also used effectively as a solute carrier.

The applied voltages tested ranged from 40 to 800 v., which provided a current flow ranging approximately from 10 to 0.5 ma.

Results

Table I outlines the essential steps by which an emetic fraction was obtained from enterotoxic culture filtrates with the respective use of saturated ammonium sulphate, 40% ethanol, phosphoric acid at pH 3.5, and the adsorbent resin "Hyflo-supercel". The final precipitate provided by each of the first three methods was shown after dialysis to be "cat-positive", i.e. on intraperitoneal injection it caused decisive vomiting, while the supernatant after removal of precipitate was inactive. An eluate from the resin was also active in cats.

TABLE I

THE CONCENTRATION OF THE EMETIC PRINCIPLE FROM BOILED ENTEROTOXIC FILTRATES OF *M. pyogenes* VAR. *aureus*, STRAIN L.16

Method and specimen	Concentrate: filtrate volume ratio	Vol. of injectant‖ (ml./kgm.)	Emetic reactions in cats	
			No. tested	No. vomiting
Control culture filtrate	1 : 1	2–5	25	25
$(NH_4)_2SO_4$ ppt'n*				
Supernatant after ppt'n (dialyzed)	1 : 1	2–5	5	0
Ppt. (dialyzed)	1 : 30	0.2–2	5	5
40% Ethanol†				
Supernatant after ppt'n (dialyzed)	1 : 1	2–5	10	0
Ppt. (dialyzed)	1 : 30–1 : 100	0.2–2	75	73
Phosphoric acid‡				
Supernatant	1 : 1	1–3	10	0
Ppt. (dialyzed)	1 : 30	1–3	13	13
Adsorbent resin§				
Heated filtrate at pH 6.35	1 : 1	1–3	5	5
Filtrate after adsorption	1 : 1	2–5	5	0
Eluate at pH 7.8	1 : 2	2–5	10	8

* *Saturated $(NH_4)_2SO_4$, 5° C., 24 hr.*

† *40% ethanol, − 20° C., 24 hr.*

‡ *Filtrate adjusted to pH 3.5 with phosphoric acid, 5° C., 24 hr.*

§ *"Hyflo-supercel" (Johns-Manville) in column.*

‖ *Reconstituted to 3 × equivalence with culture filtrate.*

A large part of the soluble material in a culture filtrate was precipitated by the boiling treatment that always preceded the use of any other method of precipitation. This initial precipitate was shown unable to cause emesis, though it did contain some alpha- and beta-lysin activity (8).

Table II depicts the step-by-step procedure adopted for extraction of the emetic factor with the use of alcohol, and lists the respective lytic and enterotoxic capacities of the various fractions of the filtrate so obtained.

TABLE II

THE DISTRIBUTION OF LYSINS AND ENTEROTOXIN IN SPECIFIC FRACTIONS OF ENTEROTOXIGENIC FILTRATES OF *M. pyogenes* VAR. *aureus*, STRAIN L.16 DURING PURIFICATION BY THE "COLD-ETHANOL" PROCEDURE

Specimen and treatment	Lysin content (titer reciprocal)			Toxic manifestations		
	α	β	δ	D.N.T.*	Lethal	Emesis
1. Uninoculated medium (Dolman)	0	0	0	0	0	0
2. Culture filtrate: unheated	64–2040	1020–2040	4	+	+	+
3. Culture filtrate: boiled 30 min.; cooled 4° C., 5 hr., centrifuged 2000 r.p.m.	0–4	4–8	0–2	0	0	+
4. Resultant precipitate from (3) resuspended	4–16	8–64	0–2	0	0	+
5. Supernatant to 40% ethanol; −20° C., 24 hr.; centrifuged 2000 r.p.m., 3 min. (temp. to remain below −10° C.)						
(*a*) Dialyzate from alcoholic filtrate	<1	<1	0	0	0	0
(*b*) Ppt. redissolved in water (10% vol.)	<1	<1	0	0	0	+
6. Undissolved ppt. 5 (*b*)	0	0	0	0	0	0
7. Reprecipitated 5 (*b*)	0.02	0.2	0	0	0	+
8. (7) Dialyzed	0.02	0.2	0	0	0	+
9. (8) Lyophilized, reconstituted	0.02	0.2	0	0	0	+
10. (9) Subjected to Durrum electrophoresis						
(*a*) Protein No. 1	0	0	0	0	0	0
(*b*) Protein No. 2	0	0	0	0	0	±†
(*c*) Protein No. 3	0	0	0	0	0	0
(*d*) Polysaccharide	0	0	0	0	0	0
11. Eluate from filter paper used in (10)	0	0	0	0	0	0

* *D.N.T. = dermonecrotoxin.*

† *Accessory signs of enterotoxicity on many occasions: emesis once.*

The staphylococcus cells and insoluble material of a culture were discarded after Seitz filtration. The filter disk was found to adsorb part of the delta-lysin content of the filtrate (8). The filtrate was then adjusted to pH 7.0 at which time it was found to contain alpha-, beta-, and delta-lysins, and possessed the properties of dermonecrotoxicity and lethality that were probably indicative of the presence of an active alpha-lysin. The filtrate was "cat-positive". After the filtrate was boiled for 30 min. and cooled to 4° C. for five hours, the resultant precipitates were recovered by centrifugation and shown to contain a small amount of active alpha- and beta-lysins.

The supernatant after removal of the precipitate was free from delta-lysin. Alpha-lysin was reduced to 0.2% and beta-lysin to 0.8% of the amount in the original filtrate. The supernatant was negative for dermonecrotoxin and was not lethal. It remained "cat-positive". After this supernatant was subjected to the alcohol treatment and centrifuged at below − 10° C., only part of the resultant precipitate was soluble in the small amount of water to which it was added (1–5 ml.). The soluble fraction was shown to contain only traces of alpha- and beta-lysins and was free from delta-lysin, but was "cat-positive". The residual precipitate, insoluble in water, was "cat-negative". The water-soluble fraction was dialyzed without causing any detectable change in its emetic properties. The active fraction was stable for at least three months at refrigerator temperatures (*ca.* 10° C.) and for a longer period, whose extent is not determined, when in the lyophilized state.

The effect of temperature on the precipitation of enterotoxin by 40% ethanol is illustrated in Table III, in which the data show that when alcohol was added to boiled culture filtrates, held at from − 5° C. to + 5° C. for 24 hr., and centrifuged at 0° C. to 10° C. the resultant precipitates were found to have suffered a progressive reduction in emetic activity in relation to the increase in temperature during precipitation.

TABLE III

THE EFFECT OF TEMPERATURE ON RECOVERY OF ENTEROTOXIN BY ETHANOL PRECIPITATION FROM BOILED FILTRATES OF *M. pyogens* VAR. *aureus*, STRAIN L.16

	Emesis from precipitates induced by ethanol				
			Occurrence of emesis		
Experiment number	Temperature during precipitation, ° C.	Final temperature after centrifuging 3 min.,° C.	No. cats tested	No. cats positive	% emesis
1.*	− 20	− 15	20	20	100
2.*	− 15	− 10	12	12	100
3.*	− 5	0	10	9	90
4.*	0	+ 6	9	6	66
5.*	+ 5	+ 10	8	2	25
6.* Supernatant from (5)	− 15 to − 20	− 10	5	5	100
7. Supernatant from (6)	− 15	− 10	5	0	0
8. Supernatant from (6), 70% ethanol	− 15	− 10	5	0	0

* *Ethanol to 40%, 12 hr.*

When the residual filtrate after removal of the precipitate obtained with alcohol at + 5° C. was kept for a further 24 hr. at −15° C. to −20° C., a second precipitate could be recovered. It was "cat-positive". The precipitate obtained during this exposure to the lower temperature apparently contained the total amount of enterotoxin that had survived boiling, since no indication of enterotoxin could be obtained from the residual filtrate after removal of this second precipitate; neither was enterotoxin present in a third precipitate obtained by increasing the ethanol to 70%, nor in the final filtrate. The

precipitate with emetic properties could be further purified by chromatographic separation but with substantial loss of the active principle. Thus, ethanol at −5° C. did not precipitate any detectable amount of enterotoxin from our culture-filtrates, but lowering the temperature to −15° C. to −20° C. allowed recovery of the greater part of the toxin.

The data in Table III also establish that if the temperature of the specimen containing the alcoholic precipitate were allowed to rise above −10° C. during centrifuging a substantial loss in activity of the precipitate resulted, presumably owing to re-solution of the enterotoxin.

More than 50 preparations of an enterotoxic concentrate, each from a different series of cultures, have been obtained by the "cold alcohol" method described. Three of these preparations have been made using strain S.6, a concentrate from which is known to cause vomiting when administered orally to monkeys (7). Specimens from all such preparations caused emesis in cats and consistently contained only small traces of lysins and were never fatal, as indicated by the representative result expressed in Table II.

The results of experiments carried out by the paper ionophoresis method of McKinley *et al.* (5) are diagrammatically expressed in Fig. 1. Their purpose was to detect the enterotoxic component of an unheated culture filtrate that had been dialyzed and concentrated by lyophilization, and to examine any possible relationship between specific lysins and enterotoxin. With the use of a Veronal buffer at pH 8.6 and after passage of 12 ma. at 200 v. for two hours four distinct fractions containing protein were detected at different distances on the anode side of the origin (proteins *A*, *B*, *C*, *D*). After elution in saline, proteins *A*, *C*, *D* were found to be hemolytic to washed sheep erythrocytes; protein *B* was not. None of them provided a positive cat-reaction.

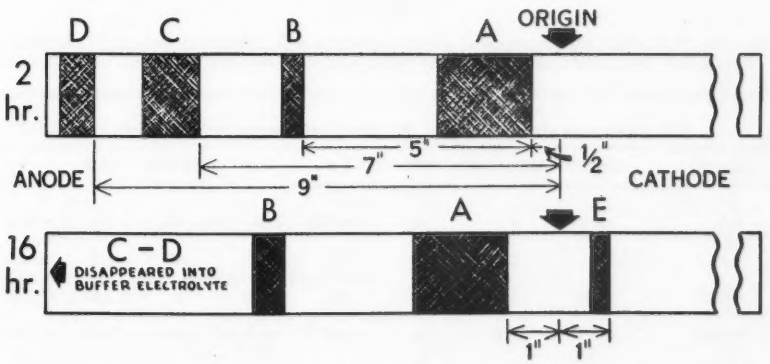


FIG. 1. A diagram of ionophoretic separation of hemolytic proteins in filtrates of *M. pyogenes* var. *aureus* by filter paper in the horizontal plane. Method after McKinley *et al.* Veronal buffer at pH 8.6; 200 v., 12 ma. Specimen was a lyophilized filtrate prepared from a medium dialyzed before inoculation. Proteins *A*, *C*, *D* were hemolytic; protein *B* was not.

After a migration period of 16 hr., proteins *C* and *D* were no longer evident and presumably had moved across the paper and into the buffer reservoir. A fifth protein fraction, *E*, was noted near the origin on its cathode side. *A* and *E* were hemolytic, but *B* was not, and again no fraction evoked a positive cat-reaction. These experiments of preliminary nature were suspended in favor of ionophoresis by the Durrum method (4) which allows the use of a larger volume of the test specimen and provides for accumulation of several milliliters of each fraction (which, however, becomes diluted with buffer solution). Table IV summarizes data from preliminary experiments made in order to determine suitable buffers and values for ionic strength, voltage, and amperage that would provide separation of the proteins present in the concentrate prepared by the "cold-alcohol" method. The method was shown to detect the pathway of flow of a protein at a concentration of 0.001% as determined with gelatine. The greatest degree of separation was found at pH 6.0. In order to confine the movement of component proteins to within the range that allowed delivery of the various fractions to the collection vessels, it was necessary to reduce the ionic strength of the buffer to 0.001 and to apply a current of 5 ma. at 700 v. These specifications were used for some 50 separations. Under these conditions concentrates of enterotoxin from strain L.16 partially purified by the "cold-alcohol" method consistently gave rise to three distinct protein fractions that moved towards the anode and a single polysaccharide fraction that showed practically no mobility. These protein fractions are referred to as proteins No. 1, 2, and 3, respectively, in order of mobility. Fig. 2 diagrammatically illustrates the principles of the equipment and the disposition of the various fractions. Comparable specimens prepared from uninoculated media provided two proteins and a carbohydrate of mobility suggestive, respectively, of proteins 1 and 3 and of the

TABLE IV

THE EFFECT OF IONIC STRENGTH OF BUFFERS UPON THE SEPARATION OF PROTEIN FRACTIONS OF CRUDE ENTEROTOXIN CONCENTRATE BY THE PAPER IONOPHORESIS METHOD OF DURRUM

Ionic strength of buffer	Resultant current		Relative degree of separation with buffers of specific pH:					
	Volts	Milliamp.	4.0	5.0	6.0	7.0	8.0	9.0
0.1	40	4	0*	0	0	0	0	0
0.01	120	2	0	0	0	0	0	0
0.001	700	5	+	+	++	±	±	±
0.0005	800	1.0	+	—	—	—	—	—
0.0005†	800	1.0	+	—	—	—	—	—

** 0 no useful separation; + effective separation; ++ most practicable degree of separation; ± partial separation with indication of secondary changes occurring within the fractions.*

† Dilute acetic acid alone.

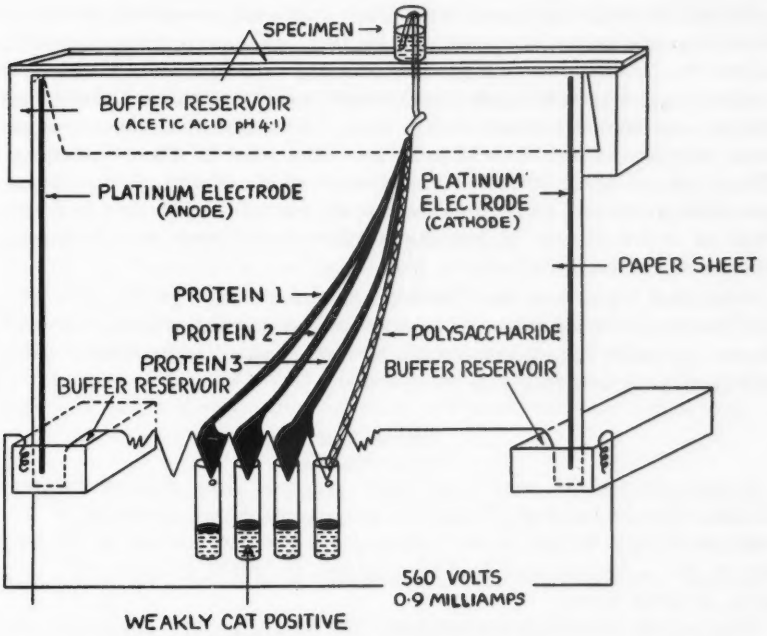


FIG. 2. A diagram of ionophoretic separation by filter paper in the vertical plane of components of a dialyzed emetic concentrate prepared from a culture filtrate of *M. pyogenes* var. *aureus*, strain L.16. Method after Durrum. Specimen was a solution of a dialyzed, partially purified precipitate obtained by the "cold ethanol" method from a culture filtrate using Dolman's medium.

TABLE V

THE RETENTION OF ENTEROTOXIN FROM CRUDE CONCENTRATES BY FILTER PAPER (S AND S 598)

Specimen	Vol. injected (ml./kgm.)	No. cats	
		Tested	Positive
1. Enterotoxin concentrate* ("cold ethanol" method)	0.5–1.5	10	10
2. Specimen (1) after suspending filter paper 2 hr., 5° C.	1–2	10	1†
3. Paper from (2) washed for 2 hr., 5° C. in:			
(*a*) Distilled water 2 hr., 5° C.	2–3	2	0
(*b*) Acetic acid pH 4.0	2–3	2	0
(*c*) Citrate–phosphate buffer pH 7.8	2–3	2	0

* *Diluted to equivalence with 20 × concentration in original filtrate.*
† *Vomited once after an interval of 76 min., no diarrhea.*

carbohydrate from the emetic specimens, but were present in greater concentration relative to the emetic preparation. The accumulated solution of protein No. 2, when injected into cats on many occasions induced the various ancillary signs associated with enterotoxicity—slight retching, and diarrhea—but decisive vomiting occurred only once. No indications of enterotoxicity were provided by solutions of proteins 1 and 3 or by the polysaccharide. This equivocal result led to an examination of the ability of filter paper to adsorb enterotoxin. Table V shows that most or all enterotoxin was absorbed from an active filtrate in two hours at room temperature. Attempts at elution were unsuccessful.

Additional trials with the Durrum apparatus using "gold-beaters' skin", parchment, and nylon fabric have since proved impracticable because of the diverse pathways for electrophoretic flow established in the former two and because of poor wettability of the nylon.

Discussion

Concentrates of an emetic principle prepared from culture filtrates of enterotoxigenic strains of *M. pyogenes* var. *aureus* have been shown to induce vomiting in cats because of the presence of a specific fraction of the filtrate that at the concentrations used for injection contained no detectable alpha-, beta-, or delta-lysins.

It should be noted that the methods used by Dack and co-workers (1, 2, 7) to obtain a partially purified concentrate that caused vomiting when fed to monkeys also yielded in our hands preparations that caused vomiting when administered to cats by the intraperitoneal route. This held true for concentrates prepared by use of ammonium sulphate, ethanol, phosphoric acid, or adsorption followed by elution from a specific resin. Similarly, a specific concentrate prepared by Dr. M. S. Bergdoll that had been shown to be "monkey-positive" and which was free from beta-lysin, caused emetic reactions in a cat that were identical with those caused by concentrates prepared in this laboratory.

Precipitation with 40% ethanol at −20° C. gave most consistent recovery of an enterotoxic concentrate, and became our method of preference for the greater part of these studies.

The enterotoxic fraction extracted from a culture filtrate by the "cold ethanol" method was separated by paper ionophoresis using the method of Durrum (4) into at least three protein and one polysaccharide fractions. Indications of enterotoxicity, not entirely unequivocal, were specifically provided by one of these proteins. Two other proteins and the polysaccharide appeared to have originated from the substance of the culture-medium. Subsequent proof that the filter paper adsorbed the toxin very retentively may well explain the weak emetic reaction noted.

A desirable precaution to observe in order to obtain maximum recovery of enterotoxin during the alcohol extraction procedure is to allow precipitation

to occur at temperatures not above −15° C. and to prevent the temperature of the preparation containing the alcholic precipitate from rising above −10° C. during centrifuging to recover the precipitate.

The Durrum method is not recommended as a means of obtaining quantities of comparatively pure enterotoxin because of the relatively small amount of solute that will move into the collecting reservoirs—a condition aggravated by the adsorption properties of the filter paper—and because this small amount is diluted by the buffer used as the solute carrier. Prolonged exposure to passage of current introduces possible problems of oxidation and heating effects and also permits the freeing into all receptor vessels of an unknown carbohydrate, apparently arising from the filter paper.

The results obtained with the apparatus, however, seem to lend support to the opinion of Bergdoll *et al.* (1) that the enterotoxin is a protein.

The electrophoretic method of McKinley *et al.* (5) seemed to offer some promise as a means of separating hemolytic proteins from a culture filtrate.

References

1. Bergdoll, M. S., Kadavy, J. L., Surgalla, M. J., and Dack, G. M. Partial purification of staphylococcal enterotoxin. Arch. Biochem. and Biophys. 33 : 259-262. 1951.
2. Bergdoll, M. S., Lavin, B., Surgalla, M. J., and Dack, G. M. Chromatography in the purification of staphylococcal enterotoxin. Science, 116 : 633-634. 1952.
3. Cremer, H. D. and Tiselius, A. Elecktrophorese von Eiweis in filtrierpapier. Biochem. Z. 320 : 272-283. 1950.
4. Durrum, E. L. Continuous electrophoresis and ionophoresis on filter paper. J. Am. Chem. Soc. 73 : 4875-4880. 1951.
5. McKinley, W. P., Maw, W. A., Oliver, W. F., and Common, R. H. The determination of serum protein fractions on filter-paper electropherograms. Can. J. Biochem. Physiol. 32 : 189-199. 1954.
6. Miller, G. L. and Golder, R. H. Buffers of pH 2 to 12 for use in electrophoresis. Arch. Biochem. 29 : 420-423. 1950.
7. Surgalla, M. J., Kadavy, J. L., Bergdoll, M. S., and Dack, G. M. Staphylococcal enterotoxin: Production methods. J. Infectious Diseases, 89 : 180-184. 1951.
8. Thatcher, F. S. and Matheson, B. H. Studies with staphylococcal toxins II. The specificity of enterotoxin. Can. J. Microbiol. 33 : 382-400. 1955.

DISTRIBUTION OF NUCLEI IN HYPHAL CELLS OF *RHIZOCTONIA SOLANI*[1]

By G. B. Sanford and W. P. Skoropad[2]

Abstract

Fixed and stained mycelium of two isolates of *Rhizoctonia solani* Kühn was examined microscopically to determine the variation in the numbers of nuclei in different cells. The number of cells examined was 568, and they were of three types. The number of nuclei in the hyphal tip cells varied from 2 to 15, the majority of cells having from four to eight. In the 'Y' type of cell the number of nuclei varied from 4 to 25, and in the majority of them from 4 to 15. The number of nuclei in the unbranched type of cell varied from 3 to 19, the majority of them having from 6 to 11 nuclei. Migration of nuclei through septal pores, in the direction of growth, was observed on several occasions *in vivo*. Difference in number of nuclei in the various living cells is a normal condition in this fungus, and the migration of nuclei through the septal pores is given as one of the possible causes of this variability.

Introduction

Among others, Hawn and Vanterpool (4) have shown that the cells of *R. solani* are multinucleate. The present study is an attempt to determine the number of nuclei in the three general types of hyphal cells of *R. solani*. Isolate No. 76, virulent on potato stems, and isolate No. 32, not pathogenic to them (8), were used for the purpose.

Methods

The modification of Maneval's (5) acid fuchsin stain was found in preliminary tests to color the nuclei so that they could be observed without difficulty under the high-power objective of the microscope. The nuclei were counted in three types of cell, namely, hyphal tip cells, 'Y' cells which were forked and bounded by septa on each of three extremities of the 'Y', and, finally, cells which were unbranched. All unbranched cells studied were located about three to five cells behind the hyphal tip cells.

The mycelium to be stained was grown on a microscope slide coated with a thin layer of potato dextrose agar. A disk of inoculum about 5 mm. in diameter was transferred to this slide and incubated in a Petri dish fitted with moist filter papers and glass supports for the glass slide. The best stains were obtained after two or three days' growth of the fungus. The nuclei were counted only in cells having a clear and definite stain for these bodies.

Results

In Table I is presented the frequency of the observed numbers of nuclei in growing tip, 'Y', and unbranched cells of isolate No. 76 and of No. 32 of *R. solani*. The number of cells was equated for the three cell types to

[1] *Manuscript received January 11, 1955.*
Contribution No. 1431 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.
[2] *Officer-in-Charge and Assistant Plant Pathologist, Plant Pathology Laboratory, c/o University of Alberta, Edmonton, Alberta.*

TABLE I

NUMBER OF NUCLEI IN CELLS OF THREE TYPES OF TWO ISOLATES OF *Rhizoctonia solani**

Number of nuclei per cell	Frequency or number of cells containing the given number of nuclei					
	Hyphal tip		'Y'		Straight	
	No. 76	No. 32	No. 76	No. 32	No. 76	No. 32
1	0	0	0	0	0	0
2	5	8	0	0	0	0
3	5	1	0	0	2	3
4	14	12	3	4	7	5
5	10	12	6	0	6	6
6	12	17	3	6	12	11
7	17	13	9	8	18	21
8	12	17	12	7	13	19
9	4	3	6	8	15	20
10	6	6	9	9	12	19
11	3	2	10	10	12	5
12	3	0	2	3	5	4
13	0	0	6	2	4	0
14	0	1	0	8	3	0
15	1	0	3	4	2	1
16	0	0	2	0	1	2
17	0	0	0	2	2	0
18	0	0	1	2	1	0
19	0	0	0	2	1	0
20	0	0	2	0	0	0
21	0	0	0	0	0	0
22	0	0	0	0	0	0
23	0	0	1	0	0	0
24	0	0	0	1	0	0
25	0	0	1	0	0	0
Totals	92	92	76	76	116	116

* *Hyphae, two to three days old.*

facilitate direct comparison of the distribution. Thus, for the hyphal tip cells, the data are based on 92 cells in each isolate. The nuclei were counted in a total of 568 cells. The size of the nucleus was approximately 2.3 μ.

The data show that the number of nuclei varies widely in each type of cell. For example, in the hyphal tip cells the range was from 2 to 15, and for the 'Y' and unbranched types of cell it was from 4 to 25, and 3 to 19, nuclei, respectively. However, in the majority of the hyphal tip cells the range was 4 to 8 nuclei, the 'Y' types 4 to 15, and the unbranched cells 6 to 11.

After several days' growth some of the hyphal cells turned yellowish in color, lost most of their cytoplasm, and, when stained, the nuclei, if present, were not visible. Also, it appeared that the older cells tended to contain fewer nuclei than the younger hyaline cells. In spite of this variability in

number of nuclei as between one cell and another in the same hypha, there was no indication of a significant difference in number of nuclei per cell characteristic of either isolate. Photographs, and also drawings, of the cells and stained nuclei in the three types of cells mentioned, and of cell anastomosis, are shown in Plate I.

Essentially identical results with respect to number of nuclei per cell, their size and general distribution, were also obtained by the use of Heidenhain's iron–alum haematoxylin stain.

At least one reason for the diversity in number of nuclei in the different cells has been given by Dowding and Bakerspigel (2), who, under the phase contrast microscope, observed nuclei of *Gelasinospora tetrasperma* to migrate from one cell to the next through the septal pores. Dowding and Buller (3), Dodge (1), and others had already concluded from their experimental data that in certain fungi the nuclei pass from one cell to another. In the present study a group of three nuclei in the cytoplasm of unstained living cells of *R. solani* was clearly observed to move very slowly from one cell to the next, and then continue into another anastomosing cell. The movement of this group of nuclei was under observation during 150 min. Similarly, one and also two nuclei in isolated masses of cytoplasm have been observed to pass slowly through the septal pore from one cell to another. Probably larger groups of nuclei migrate from one cell to another. The movement began sporadically and was always in the direction of growth of a new cell or toward one anastomosing with another.

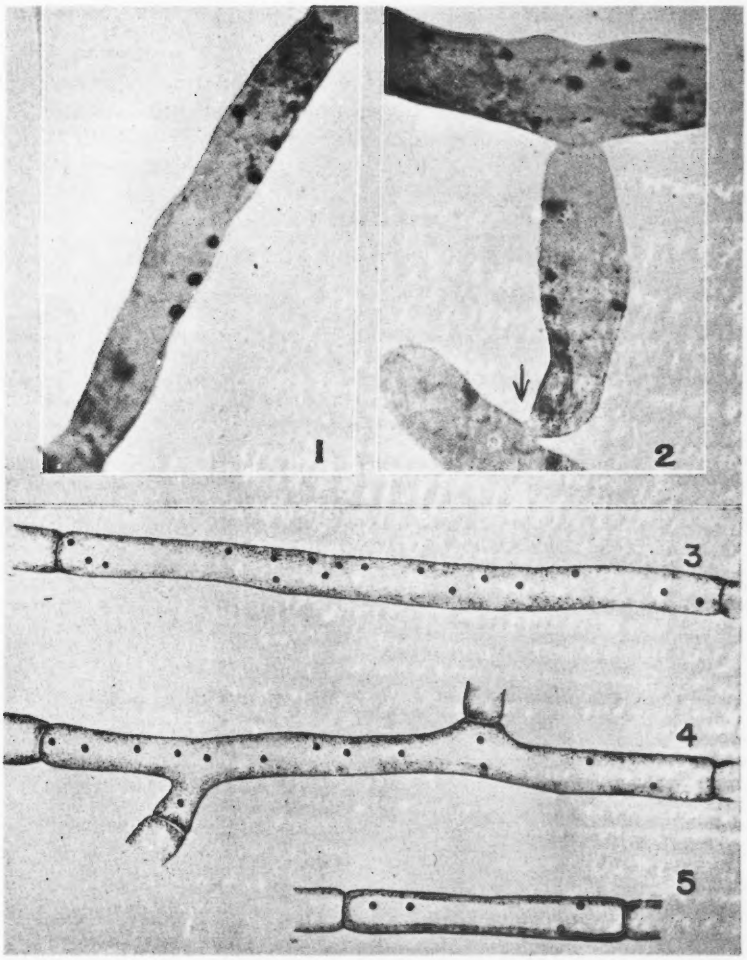
Discussion

Hyphal anastomosis between cells of the same and different isolates is extremely common and variable in *R. solani* and has occurred under a wide range of conditions in the laboratory. Pontecorvo (6), 1946, states, "That nuclei from one hypha can migrate to another following hyphal fusion has been shown again and again in many species of *Fungi imperfecti*, as well as in species having a sexual stage." In the same article he also states, ". . . . the nuclei of multinucleate cells in *Fungi imperfecti* are therefore not synchronized and may divide at different rates." Different rates of division are not necessarily assumed in the present paper.

Pontecorvo (7) concluded in 1953, from experimental data that, "Genetic recombinations can be obtained outside the sexual cycle, and this has already been done with the asexual species *Aspergillus niger*." This statement was based on somatic reduction to the haploid condition in some of the diploid nuclei, with or without recombination between non-homologous chromosomes; somatic crossing-over as outlined by Stern (9); and either mutation or crossing-over between closely linked markers.

Further discussion of other genetical and physiological mechanisms that may operate to determine the numbers and kinds of nuclei in different cells of *R. solani* at any given time seems unnecessary in this paper. Let it suffice to

PLATE I



FIGS. 1 and 2. Photomicrographs, with oil-immersion objective, of stained nuclei (av. 2·3 μ) in three-day-old hyphal cells of *Rhizoctonia solani*. 1. Unbranched cell with eight nuclei plainly visible, also septal pores of cells. 2. Anastomosing hyphae with stained nuclei.

FIGS. 3, 4, and 5. Camera lucida drawings of observed nuclei in stained hyphal cells of *Rhizoctonia solani*. 3. Unbranched cell, three days old, 17 nuclei. 4. Branched cell, three days old, 14 nuclei. Unbranched cell, 10 days old, four nuclei.

say that the genetical possibilities arising from the migration of nuclei from cell to cell in *R. solani* in the same mycelium as well as in frequent anastomosis between pathogenically different races of this fungus in nature would have much practical significance.

Acknowledgments

The authors are grateful to Mrs. A. H. H. Grinchenko, assistant technician, for helping to count the nuclei and for the camera lucida drawings; and to Dr. C. O. Person, Department of Plant Science, University of Alberta, for taking the photographs.

References

1. DODGE, B. O. Heterokaryotic vigor in *Neurospora*. Bull. Torrey Botan. Club, 69 : 75-91. 1942.
2. DOWDING, E. S. and BAKERSPIGEL, A. The migrating nucleus. Can. J. Microbiol. 1 : 68-78. 1954.
3. DOWDING, E. S. and BULLER, A. H. R. Nuclear migration in *Gelasinospora*. Mycologia, 32 : 471-488. 1940.
4. HAWN, E. J. and VANTERPOOL, T. C. Preliminary studies on the sexual stage of *Rhizoctonia solani* Kühn. Can. J. Botany, 31 : 699-710. 1953.
5. MANEVAL, W. E. Staining bacteria and yeasts with acid dyes. Stain Technol. 16 : 13-19. 1941.
6. PONTECORVO, G. Genetic systems based on heterocaryosis. Cold Spring Harbor Symposia Quant. Biol. 11 : 193-201. 1946.
7. PONTECORVO, G. (*With sections by* Roper, J. A., Hemmons, L. M., Macdonald, K. D., and Bufton, A. W. J.). The genetics of *Aspergillus nidulans*. Advances in Genet. 5 : 141-238. 1953.
8. SANFORD, G. B. Persistence of *Rhizoctonia solani* Kühn in soil. Can. J. Botany, 30 : 652-664. 1952.
9. STERN, C. Somatic crossing-over and segregation in *Drosophila melanogaster*. Genetics, 21 : 625-730. 1936.

SOME EFFECTS OF POSTIRRADIATION TREATMENT WITH METABOLIC INHIBITORS AND NUTRIENTS UPON ULTRAVIOLET IRRADIATED SPORES OF *STREPTOMYCES* T12[1]

By S. D. Wainwright[2] and Ann Nevill

Abstract

The effects of postradiation treatment with metabolic inhibitors (arsenate, azide, and dinitrophenol) and various cell nutrients (N-sources and sugars) upon ultraviolet irradiated spores of *Streptomyces* T12 have been studied. Changes in the frequency of induced variants which were not accompanied by effects upon survival, and increases of survival which were not associated with a change in variant frequency were observed. The results obtained indicate that the biological effects of ultraviolet radiation can be modified by the postradiation metabolism of the cell.

Introduction

We have recently demonstrated that postradiation treatment of ultraviolet irradiated spores of *Streptomyces* strain T12 in distilled water, with iodoacetate, or with a peptone medium can modify both the extent of survival and the proportion of radiation-induced heritable modifications of character. Some portion, at least, of the lethal action of the radiation can be modified without any accompanying change in the frequency of induced variants (13, 14).

We have now examined the effects of other agents selected on the basis of similarity of chemical structure to that of iodoacetate and/or known effects upon cell metabolism. The results indicate that the modifications of the effects of ultraviolet radiation by postradiation treatments were not due to any relatively simple non-metabolic chemical reaction.

Material and Methods

All experiments were performed with suspensions of spores of *Streptomyces* strain T12 freshly prepared from plates inoculated with standard suspensions and incubated at 28° C. for five days. The spores were suspended in distilled water, agitated in a blendor, and filtered as previously described (13). For experiments with washed suspensions, after filtration the spores were spun down, washed once by centrifugation, and resuspended in sterile distilled water.

A General Electric germicidal lamp was used for the irradiation, and doses were measured with a Westinghouse dose meter. A standard dose of 400

[1] *Manuscript received December 31, 1954.*

Contribution from the Biology Branch, Atomic Energy of Canada, Ltd., Chalk River, Ontario. Issued as A.E.C.L. No. 173.

[2] *National Research Council (Canada) Fellow 1952-53. Atomic Energy of Canada, Ltd. Fellow 1953-54. Present address: Department of Microbiology, Yale University, New Haven, Connecticut, U.S.A.*

ergs/mm.^{2} was used except where otherwise indicated. All operations subsequent to the irradiation were performed by the light of red lamps to prevent photoreactivation.

Four-milliliter samples of irradiated suspension were diluted with 1 ml. of either distilled water or reagent solution, incubated at 37° C., usually for three hours, and plated on a standard medium containing: 0.5 gm. K_2HPO_4, 0.5 gm. asparagine, 0.5 gm. "Bacto" peptone, 15 gm. agar, and water to 1 liter. After dilution in the suspension, the peptone medium used in the experiments was of the same composition as the standard medium except that glucose and agar were omitted. Reagent solutions were adjusted to pH 7.2.

Estimates of the proportions of survivors and of induced variant colonies were made from 10 replicate plates incubated for six to seven days at 28° C. as described previously (13). Thus, most estimates of the frequency of variants are based upon totals of between 100 and 800 colonies. However, the relatively large standard deviation of any one estimate is more than compensated for by the high frequency of variants, which makes it possible for many *separate* replicate experiments to be performed.

Results

Effects of Sodium Arsenate

Postradiation treatment with arsenate caused a consistent, small increase in the proportion of induced morphologically variant colonies from spores irradiated at a dose of 400 ergs/mm.^{2} (Table I: χ^2 for pooled data 70.84, $P < 0.001$). In most of the experiments arsenate treatment also caused a small increase in survival.

The increased proportion of variant colonies was due to an increase in the frequency of heritable changes rather than the induction of phenocopies. Fifty-two individual variant colonies were isolated from suspensions treated with arsenate, and all were found to retain their variant character upon subculture. If the increased variant frequency had been due to the induction of temporary physiological variations of character, some ten of these isolates would have been expected not to subculture as variant strains (P for the observed result < 0.001).

Treatment with arsenate did not affect the survival of control unirradiated spores or the proportion of spontaneous variants, which was of the order of 1-2% ($P > 0.8$ for 36,000 colonies).

A concentration of 2×10^{-4} M arsenate was somewhat more effective in modifying the actions of ultraviolet radiation than concentrations of 2×10^{-3} M and 2×10^{-5} M, but the differences between the effects of the various concentrations were not significant.

TABLE I

EFFECTS OF POSTRADIATION TREATMENT WITH ARSENATE UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY

Expt. No.*	Ratio of viable counts Treated/Control	Ratio of variant frequencies Treated/Control
1	4.07	1.59
2	1.19	1.08
3	1.54	1.48
4	0.95	1.17
5	1.69	1.22
6	1.76	1.07
7	2.07	1.22
8	1.56	1.03
9	1.62	1.22
10	1.14	1.22

NOTE: *Irradiated spores were treated for three hours at 37° C. in distilled water (control) or* 2×10^{-4} *M sodium arsenate (treated).*

* *Experiments 1–5 were with washed spore suspensions: experiments 6–10 with unwashed suspensions.*

Some experiments were performed with washed spore suspensions, but the effects were not consistently more marked nor more quantitatively reproducible than with unwashed suspensions (Table I).

The effects of postradiation treatment with arsenate were similar to the effects of a corresponding treatment with iodoacetate previously reported (13), but were never as marked as the maximal effects of iodoacetate treatment. Both arsenate (Table I) and iodoacetate (13) caused an increased variant frequency and increased survival among spores irradiated at a dose of 400 ergs/mm.^{2} In the presence of peptone medium the effects of postradiation treatment with arsenate upon both the proportion of survivors and the variant frequency were suppressed in the same manner as the corresponding effects of treatment with iodoacetate (13). Among spores irradiated at a dose of 200 ergs/mm.^{2} postradiation treatment with either arsenate (Table II) or iodoacetate (13) resulted in a slightly greater proportion of

TABLE II

EFFECTS OF POSTRADIATION TREATMENT WITH ARSENATE UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY WITH SPORES IRRADIATED AT 200 ERGS/MM.^{2}

Expt. No.	Proportion of survivors $\times 10^2$		Variant frequency (%)	
	Control	Treated	Control	Treated
1	3.6	4.2	36.6 ± 4.0	37.0 ± 3.3
2	3.4	4.3	41.2 ± 4.2	38.3 ± 3.7
3	3.5	4.6	31.8 ± 3.1	31.7 ± 2.7

NOTE: *Irradiated spores were treated for three hours at 37° C. in distilled water (control) or* 2×10^{-4} *M sodium arsenate (treated).*
These experiments were performed with washed spore suspensions.

survivors than among control irradiated spores similarly treated in distilled water, but caused no change in the variant frequency.

A further parallel between the effects of postradiation treatments with arsenate and with iodoacetate (13) was found with spores subjected to a shorter period of postradiation treatment. Treatment of spores irradiated at 400 ergs/mm.^{2} for only 15 min. with 2×10^{-4} M arsenate caused a small increase in the frequency of variant colonies above that observed for control spores similarly treated in distilled water (χ^2 for pooled data of a series of 24 consistent experiments 66.58, $P < 0.001$). Treatment with arsenate produced no detectable effects upon the proportions of surviving spores, except in one experiment in which there was a reduction to 50% of the proportion observed when spores were treated in distilled water.

As in the case of spores treated for longer periods, the increase in variant frequency was not due to the induction of phenocopies. Two hundred and forty individual colonies were isolated from suspensions treated with arsenate in this series of experiments and all retained their variant character on subculture. If the increased variant frequency had been due solely to temporary physiological modifications of character, some 55 of these isolates would have been expected to subculture as non-variants ($P < 0.001$).

The increased variant frequency due to the presence of arsenate during the 15-min. period of postradiation treatment could not be accounted for solely by effects of the concentrations of arsenate expected to be carried over to the medium during plating. The variant frequencies for suspensions treated with arsenate and plated on standard medium were significantly higher than for suspensions treated in distilled water and plated on standard medium containing an equivalent concentration of arsenate (10^{-7}–10^{-6} M) (χ^2 for pooled data of eight consistent experiments 7.75, $P < 0.006$). Nevertheless, the presence in the plating medium of as small a concentration of arsenate as 10^{-7} M did cause a slight, but significant increase in variant frequency (χ^2 for pooled data of 12 consistent experiments 6.97, $P < 0.01$).

Postradiation treatment with arsenate has also been found to cause increased survival of ultraviolet irradiated cells of *Escherichia coli* (6).

Effects of Sodium Azide and Dinitrophenol

Postradiation treatment with azide caused an increase in the proportion of spores surviving doses of 400 ergs/mm.^{2} (Table III) and 200 ergs/mm.^{2} above that observed with control spores in distilled water. This increased survival was not associated with modifications of the variant frequency, except in one experiment. The results of the series of experiments showed no significant effect either by the sign test or the χ^2 value for the pooled data. No effects of azide treatment were found with concentrations of 2×10^{-4} M

TABLE III

EFFECTS OF POSTRADIATION TREATMENT WITH AZIDE UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY

Expt. No.	Proportion of survivors $\times 10^6$		Variant frequency (%)	
	Control	Treated	Control	Treated
1	40	202	45.5 ± 2.4	40.9 ± 3.3
2	662	863	37.3 ± 2.2	32.3 ± 2.3
3	386	436	41.2 ± 4.2	41.2 ± 3.8
4	23	37	43.6 ± 3.2	49.2 ± 2.6
5	46	58	30.1 ± 2.1	41.9 ± 2.0
6	40	69	39.8 ± 2.1	36.4 ± 4.9

NOTE: *Irradiated spores were treated for three hours at 37° C. in distilled water (control) or* 2×10^{-3} *M sodium azide (treated).*

or less. Treatment with 2×10^{-3} *M* azide did not affect the survival of control unirradiated spores or the frequency of spontaneous variants ($P > 0.6$ for 24,800 colonies).

The increased survival resulting from postradiation treatment with 2×10^{-3} *M* azide was suppressed in the presence of peptone medium, and the effects of the combined treatment upon both the proportion of survivors and the variant frequency were the same as the effects of the medium alone. This result suggests that the failure of Berger, Haas, Wyss, and Stone (1) to find effects of postultraviolet radiation treatment with azide upon cells of *E. coli* may have been due to the effects of nutrients released into the medium from the irradiated cells (3).

Postradiation treatment with peptone medium inhibits the increases in both the proportion of survivors and the variant frequency which occur during postradiation treatment in distilled water (13). Dinitrophenol reversed this inhibitory effect of peptone medium upon the increase in variant frequency, but did not affect the inhibitory action of peptone medium upon the increase in proportion of survivors (Table IV). However, treatment with dinitrophenol in the absence of peptone medium did not significantly modify either the proportion of survivors or the variant frequency (χ^2 for pooled data 1.58, $P > 0.2$) obtained with control spores incubated in distilled water. Treatment with dinitrophenol was also without effect upon the survival of control unirradiated spores or upon the frequency of spontaneous variants ($P > 0.6$ for 21,000 colonies).

Similar results were obtained with concentrations of dinitrophenol of 2×10^{-4} *M* and 10^{-4} *M*.

Postradiation treatment with dinitrophenol has also been observed to affect ultraviolet irradiated cells of *E. coli* when the agent was added to the plating medium. Under these conditions, both the proportion of survivors and the frequency of induced streptomycin-resistant mutants were increased (6).

TABLE IV

EFFECTS OF POSTRADIATION TREATMENT WITH DINITROPHENOL UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY

Expt. No.	Controls (distilled water)		Treated with 2×10^{-3} *M* dinitrophenol	Treated with medium	Treated with medium and dinitrophenol
	0 hr.	3 hr.			
(a) Variant frequency (%)					
1	58.9 ± 1.8	72.0 ± 2.9	76.1 ± 3.1	58.3 ± 4.8	67.5 ± 4.4
2	43.8 ± 1.8	51.0 ± 3.9	53.3 ± 3.5	35.6 ± 4.2	50.3 ± 4.6
3	16.9 ± 1.5	23.4 ± 1.4	25.4 ± 1.5	14.8 ± 1.4	20.3 ± 1.7
(b) Proportion of survivors $\times 10^6$					
1	6	18	16	8	9
2	5	12	14	9	8
3	49	71	71	49	52

NOTE: *Spores irradiated at a dose of 400 ergs/mm.² were treated for three hours at 37° C. in distilled water (three hour control) or with added reagent. Samples were also plated immediately after the irradiation (zero hour control).*

Effects of Nitrogen Sources and Carbohydrates

Further study has shown that the effects of postradiation treatment with peptone medium (13) involve two distinct processes. Inhibition of the increases in proportion of survivors and in variant frequency which occur during treatment in distilled water have also been observed with 0.05% (w/v) peptone alone. However, suppressal of the effects of postradiation treatment with iodoacetate has only been obtained with the complete peptone–phosphate–asparagine medium. In the present study we have been concerned only with the activities of N-sources as inhibitors of the effects of postradiation incubation in distilled water.

An acid hydrolysate of casein ("Bacto Casamino acids") was tested at two concentrations and found to inhibit the increases in both the proportion of survivors and the induced variant frequency which occur in distilled water. The higher concentration (0.2% w/v) roughly corresponded to the peptone medium in concentration of total nitrogen. Results for the lower concentration (0.08% w/v) are given in Table V. Similar results were obtained with ammonium chloride at a concentration of 0.5% (w/v).

Sodium nitrate was tested at a concentration of 0.2% (w/v) and found to be without significant effect upon either the proportion of survivors or the induced variant frequency. This concentration of nitrate corresponds to that present in Czapek-Dox medium, which supports excellent growth of the T12 strain of *Streptomyces*.

We have previously found (13) the effects of postradiation treatment with 10^{-3} *M* arabinose for three hours to be inconsistent. The inconsistency was in the effect upon survival, for the differences in variant frequencies between

TABLE V

EFFECTS OF POSTRADIATION TREATMENTS WITH N-SOURCES UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY

Expt. No.	Proportion of survivors × 10^7			Variant frequency (%)		
	0 hr. control	3 hr. control	Treated	0 hr. control	3 hr. control	Treated
1*	233	400	310	27.2 ± 2.6	39.7 ± 2.2	32.7 ± 2.4
2*	54	144	71	48.5 ± 2.2	58.8 ± 4.0	42.6 ± 1.9
3*	98	184	100	47.5 ± 4.5	51.7 ± 3.3	43.6 ± 4.4
4*	31	62	41	40.9 ± 2.6	48.7 ± 1.8	44.0 ± 2.2
5†	37	77	57	28.6 ± 1.7	42.6 ± 4.0	31.3 ± 4.4
6†	28	42	27	41.7 ± 2.7	44.3 ± 2.3	40.1 ± 2.8
7†	55	134	58	36.1 ± 2.0	53.4 ± 4.2	39.7 ± 2.0

NOTE: *Spores irradiated at a dose of 400 ergs/mm.² were incubated for three hours at 37° C. in distilled water (three hour control) or with N- source (treated). Samples were also plated immediately after irradiation (zero hour control).*

* *In experiments 1–4 the N- source was 0.08% (w/v) casein hydrolysate.*

† *In experiments 5–7 the N- source was 0.5% (w/v) ammonium chloride.*

suspensions treated with arabinose and those treated in distilled water were not significant. The differences in proportions of survivors following the arabinose and control water treatments were small, and did not exceed 20%. However, they appear to be a genuine reflection of small effects of treatment with arabinose, for a similar inconsistency of effect upon survival has also been found under conditions where a consistent significant effect upon the variant frequency was obtained (Table VI). In these experiments the

TABLE VI

INFLUENCE OF BRIEF POSTRADIATION TREATMENT WITH ARABINOSE UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY

Expt. No.	Proportion of survivors $\times 10^6$		Variant frequency (%)		P value for difference in variant frequency
	Control (distilled water)	Treated (arabinose)	Control (distilled water)	Treated (arabinose)	
1	8	19	28.2 ± 3.6	10.3 ± 2.1	< 0.001
2	15	21	29.8 ± 2.6	21.4 ± 2.8	< 0.05
3	11	22	55.0 ± 2.9	37.4 ± 2.0	< 0.001
4	45	49	28.4 ± 2.9	19.8 ± 2.2	< 0.02
5	79	98	40.3 ± 2.4	32.7 ± 2.0	< 0.01
6	50	118	37.4 ± 3.5	24.7 ± 2.0	< 0.01
7	6	7	37.0 ± 4.4	35.3 ± 5.6	$0.8 < P < 0.9$
8	15	15	34.5 ± 3.4	26.9 ± 2.8	$0.05 < P < 0.10$
9	38	32	47.6 ± 2.0	45.7 ± 2.4	$0.3 < P < 0.5$
10	125	125	19.4 ± 1.4	16.5 ± 1.2	$0.05 < P < 0.10$

NOTE: *For details see text.*

proportions of survivors and the variant frequencies were compared after postradiation treatment in distilled water and with 2×10^{-4} M arabinose for only 15 min. Arabinose treatment consistently resulted in a lower variant frequency, but did not consistently modify the proportion of survivors. Actually, the experiments appeared to fall into two groups; those (Nos. 1–6) in which a significant increase in survival was accompanied by a marked decrease in variant frequency, and those (Nos. 7–10) in which there was no significant effect upon survival but a small decrease in variant frequency (χ^2 for pooled data 9.915, $P < 0.002$). There were no detectable effects arabinose treatments upon the survival of control unirradiated spores or upon the frequency of spontaneous variants. Similar results have also been obtained with 2×10^{-4} M glucose.

We have also examined the effects of postradiation treatment for three hours with maltose and lactose (both sugars recrystallized and N-free) at concentrations of 2×10^{-4} M and 10^{-4} M. Neither sugar significantly affected either the proportion of survivors or the frequency of induced variants.

Arabinose, glucose, and maltose, when present as sole carbon source in a Czapek-Dox medium, supported growth of *Streptomyces* T12 but lactose did not support growth.

Effects of Potassium Phosphate, Sodium Acetate, and Sodium Sulphate

Postradiation treatment with phosphate buffer (pH 7.2) caused a slightly greater increase in survival than treatment in distilled water (Table VII). This effect was accompanied by a small, but consistent, reduction in the proportion of induced variants (χ^2 for pooled data 21.42, $P < 0.001$).

TABLE VII

EFFECTS OF POSTRADIATION TREATMENT WITH PHOSPHATE UPON THE PROPORTION OF SURVIVORS AND THE VARIANT FREQUENCY

Expt. No.	Proportion of survivors $\times 10^7$		Variant frequency (%)	
	Control	Treated	Control	Treated
1	43	55	44.2 ± 2.3	36.5 ± 2.0
2	42	47	50.7 ± 2.1	39.0 ± 2.0
3	43	52	52.1 ± 2.7	45.5 ± 2.4
4	85	89	48.4 ± 1.9	45.6 ± 1.8

NOTE: *Irradiatedspores were treated for three hours at 37° C. in distilled water (control) or with* 2×10^{-4} *M potassium phosphate buffer (pH 7.2) (treated).*

Results obtained after combined treatment with phosphate and an equimolar concentration of sodium arsenate were complex. The proportion of survivors was greater than after treatment with either arsenate or phosphate alone. On the other hand, the frequency of induced variants was less than after treatment with arsenate alone, but greater than after treatment with phosphate alone.

The effects of 2×10^{-4} *M* concentrations of acetate and sulphate were examined, and neither agent was found to affect significantly either the proportion of survivors or the variant frequency. Acetate, when present as the sole carbon source in a Czapek-Dox medium, would not support the growth of *Streptomyces* T12.

Discussion

(*a*) *The Contribution of Effects upon Growth to the Observed Effects of Postradiation Treatments*

It is very improbable that the effects observed after postradiation treatments could be attributed to nuclear divisions and growth followed by subsequent fragmentation of the resulting mycelium during the plating. Cytological studies have shown that even after unirradiated spores are incubated in a complete medium at 37 °C. for three hours formation of the germination tube is barely initiated. This possibility of growth and fragmentation is even less likely when other reagents are used, because cytological examination (14) has shown that less than 2% of unirradiated spores germinate during the course of incubation for five days without added medium.

It is equally improbable that the observed changes in variant frequencies were due to effects (caused by reagent carried over during plating) upon the germination and growth on the plates resulting in the selection of variant or non-variant types of growth. In the case of arsenate we have demonstrated that the expected concentrations transferred to the medium are inadequate to account for observed effects of the treatment. In the cases of ammonium chloride and casein hydrolysate, the results parallel those with peptone, which together with phosphate and glucose (which can be replaced by arabinose) is normally present in the medium in much higher concentrations than those carried over during the plating. Treatment with the other agents tested did not modify the frequencies of induced variants.

(*b*) *Mechanism of Action of the Agents*

Two general types of mechanism can be proposed to account for the effects of treatments with chemical agents upon ultraviolet irradiated cells, and at the present time it is not possible to discriminate critically between them (13). Therefore, we have been principally concerned in determining whether the observed effects are due primarily to modifications of the postradiation spore metabolism, or primarily to *direct* participation in some simple reaction chain involving a rather specific type of radiation-produced "poison" (e.g. organic peroxides).

Agents most effective in modifying the proportion of survivors include iodoacetate, azide, and ammonium ions which differ markedly in chemical structure, whereas the least effective compounds are more closely related to iodoacetate in structure. Thus, the results obtained in this survey would require an increase in the complexity of the set of postulates suggested as the minimum requirements needed to interpret previous results on the basis of a simple chemical mechanism (13, 14).

On the other hand, our findings can readily be attributed to effects upon the postradiation metabolism of the spores. From the extensive information available in the literature (4, 9, 11) concerning the biochemical actions of the reagents used, and from the observed effects upon survival, it is possible to make a tentative identification of the major lethal action of the radiation as a derangement of the balance of cell metabolism.

Detailed speculation is clearly not warranted at this juncture. Nevertheless, we wish to note that the two principal features of the induced metabolic imbalance are compatible with the scant information currently available. The major feature would be an impairment of the synthesis of desoxyribonucleic acid, which has been observed to be a most marked effect of ultraviolet radiation (7, 8, 10). The second feature would be an undiminished (or even elevated) rate of generation of energy in a form immediately available for key synthetic processes. There is a paucity of relevant information concerning this point, but it is known that the rates of growth, ribonucleic acid synthesis, and oxygen consumption are not immediately and markedly affected (7, 8, 10). Further, in the case of X-irradiated cells of *Escherichia coli*, "leakage" of

compounds containing energy-rich phosphate bonds into the medium has been observed (2). It is also highly significant that a similar scheme of "unbalanced syntheses" recently has been postulated by Cohen and Barner (3) from the observations that thymine and glucose could cause transient increases in survival of ultraviolet irradiated cells of strains of *E. coli* similar to those found after treatment with α methyl-glucoside (12). A somewhat different scheme of "unbalanced syntheses" has also been proposed by Heinmets, Lehman, Taylor, and Kathan (5).

The induction of heritable changes of character would be expected to be determined by a more subtle disturbance of cell metabolism than gross lethal derangements. Thus, it is not surprising that the effects of postradiation treatments upon the frequencies of induced variants have not paralleled the effects upon survival for most of the agents tested.

The observed effects were relatively small in proportion to the effects of the radiation. However, it must be borne in mind that it is not possible to determine the effects of a radiation upon microbial cells without applying some form of posttreatment such that metabolism and, usually, growth may occur. Thus, the effects which we have studied are those of the agent tested superimposed upon the effects of subsequent incubation on a nutrient medium. Hence, the extent to which the effects of the radiation may be modified by the postradiation metabolism of the spore may be considerably greater than that revealed by these studies.

References

1. BERGER, H., HAAS, F. L., WYSS, O., and STONE, W. S. Effect of sodium azide on radiation damage and photoreactivation. J. Bacteriol. 65 : 538-543. 1953.
2. BILLEN, D., STREHLER, B. L., STAPLETON, G. E., and BRIGHAM, E. Postirradiation release of adenosine triphosphate from *Escherichia coli* B/r. Arch. Biochem. and Biophys. 43 : 1-10. 1953.
3. COHEN, S. S. and BARNER, H. D. Studies on unbalanced growth in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. 40 : 885-893. 1954.
4. FRUTON, J. S. and SIMMONDS, S. General biochemistry. John Wiley & Sons, Inc., New York. 1953.
5. HEINMETS, F., LEHMAN, J. J., TAYLOR, W. W., and KATHAN, R. H. The study of factors which influence metabolic reactivation of the ultra-violet inactivated *Escherichia coli*. J. Bacteriol. 67 : 511-522. 1954.
6. JACOBS, L. Private communication.
7. KANAZIR, D. and ERRERA, M. Métabolisme des acides nucléiques chez *E. coli* B après irradiation ultraviolette. Biochim. et Biophys. Acta, 14 : 62-66. 1954.
8. KELNER, A. Growth, respiration, and nucleic acid synthesis in ultraviolet-irradiated and in photoreactivated *Escherichia coli*. J. Bacteriol. 65 : 252-262. 1953.
9. LARDY, H. A. and WELMAN, H. The catalytic effect of 2, 4 dinitrophenol on adenosine triphosphate hydrolysis by cell particles and soluble enzymes. J. Biol. Chem. 201 : 357-370. 1953.
10. SIMINOVITCH, L. Biochemical modifications of the bacterial host during bacteriophage development. Ann. inst. Pasteur, 84 : 265-272. 1953.
11. UMBREIT, W. W. Metabolic maps. Burgess Publishing Company, Minneapolis, Minnesota. 1952.
12. WAINWRIGHT, S. D. and MULLANEY, J. Some effects of metabolic inhibitors upon survival of ultra-violet irradiated *Escherichia coli*. Experientia, 9 : 376-377. 1953.
13. WAINWRIGHT, S. D. and NEVILL, A. Modification of the biological effects of ultra-violet irradiation by post-radiation treatment with iodoacetate and peptone. J. Gen. Microbiol. 12 : 1-12. 1955.
14. WAINWRIGHT, S. D. and NEVILL, A. Induction of provariants as a function of dose of ultraviolet radiation. J. Gen. Microbiol. 12 : 13-24. 1955.

RELATIONSHIPS BETWEEN POSTRADIATION TREATMENTS WITH IODOACETATE AND LIGHT[1]

By S. D. Wainwright[2] and Ann Nevill

Abstract

When ultraviolet irradiated spores of *Streptomyces* T12 were treated with iodoacetate for periods of up to six and a half hours and then photoreactivated, the proportion of survivors was greater than that for control spores similarly treated in distilled water. The frequencies of variant colonies from spores treated with iodoacetate and photoreactivated did not differ significantly from those obtained when spores were similarly treated in distilled water. The principal effect of iodoacetate treatment was inhibition of the loss of photoreversibility, as measured by the effect upon proportion of survivors.

Introduction

The lethal and mutagenic effects of ultraviolet radiation can be modified both by exposure to visible light and by other appropriate postradiation treatments. However, the effects of iodoacetate and other chemicals upon ultraviolet irradiated spores of *Streptomyces* T12 differ from those obtained by exposure to visible light (3, 4, 5). Jacobs (2) has, similarly, observed effects of postradiation treatments with chemical agents upon ultraviolet irradiated cells of *Escherichia coli*, and also effects of certain of the agents upon the level of survival observed among photoreactivated cells. It therefore seemed of interest to study the relationships between the effects of iodoacetate and visible light upon ultraviolet irradiated spores of *Streptomyces* T12. This communication reports the results obtained.

Methods

Methods used in preparing the spore suspensions of *Streptomyces* strain T12 and the majority of the experimental methods are given in the accompanying paper (5).

Unless otherwise stated, a standard dose of 400 ergs/mm.^{2} of ultraviolet radiation was used.

A General Electric air-cooled mercury lamp (Type B—H6) delivering a flux of 60,000 lumens per sq. ft. was the source of visible light. The lamp was separated from a water bath maintained at 28 $\pm$ 1° C. by a 2-mm. clear glass filter. A pyrex standard test-tube of 15 mm. diameter containing 3 ml. of suspension was placed in the bath with the wall approximately 3 cm. from the lamp. Unless otherwise stated, suspensions were irradiated for a standard period of 10 min. and then returned to the "dark" (light of red lamps), as this exposure was inadequate for maximum photoreactivation (see Results).

[1] *Manuscript received December 31, 1954.*
Contribution from the Biology Branch, Atomic Energy of Canada Ltd., Chalk River, Ontario. Issued as A.E.C.L. No. 172.
[2] *Atomic Energy of Canada Ltd. Fellow 1953–54. Present address: Department of Microbiology, Yale University, New Haven, Connecticut, U.S.A.*

All experiments contained appropriate controls consisting of irradiated spores treated in distilled water.

Results

Effects of Treatments with Iodoacetate and Light upon Survival

When ultraviolet irradiated spores were incubated with iodoacetate for three hours and then photoreactivated the proportion of survivors was greater than among controls (Table I, columns 3 and 5). As observed previously (3, 4), the proportion of survivors was greater among non-photoreactivated spores treated with iodoacetate than with corresponding controls. However, the extent of photorevival [3] (i.e. ratio of survival after photoreactivation to survival among non-photoreactivated spores) was greater in the case of iodoacetate-treated spores than for the controls.

TABLE I

EFFECTS UPON PROPORTION OF SURVIVORS AND FREQUENCY OF VARIANT COLONIES OF TREATMENT FOR THREE HOURS BEFORE PHOTOREACTIVATION

		Spores treated for 3 hr. at 37° C. with			
		Distilled water		Iodoacetate	
Expt. No.	Untreated unphotoreactivated control* (1)	Control (2)	Photoreactivated (3)	Control (4)	Photoreactivated (5)
(a) Proportion of survivors $\times 10^6$					
1	27	31	11,200	58	30,000
2	4.3	9.9	5,500	32	24,500
3	11	21	2,440	51	7,500
(b) Frequency of variants (%)					
1	12.5 ± 1.4	18.6 ± 1.5	37.0 ± 3.2	33.7 ± 4.3	37.0 ± 1.9
2	32.8 ± 1.9	33.7 ± 4.1	38.6 ± 1.8	51.1 ± 2.4	41.2 ± 2.7
3	28.3 ± 3.4	39.6 ± 2.7	47.9 ± 2.7	44.3 ± 1.7	47.1 ± 4.5

NOTE: *Irradiated spores were treated at 37° C. in distilled water and with* 2×10^{-4} *M sodium iodoacetate (pH 7.2). Samples were plated without and after photoreactivation.*

* *Spores plated immediately after ultraviolet radiation and dilution of the suspension.*

[3] *Our results indicate that the effects of exposure to visible light upon survival differ in many respects from the effects upon the frequency of induced variant colonies. For clarity of presentation, we therefore propose to term the increased survival the photorevival, and the change in the frequency of variant colonies will be termed photorestoration. Similarly, where appropriate, the term photoreversibility (i.e. property of responding to exposure to visible light by increased survival and changed frequency of variant colonies) will be replaced by the terms photorevivability and photorestorability, respectively.*

Conversely, when irradiated spores were photoreactivated and then incubated with iodoacetate the proportion of survivors was higher than for control spores (Table II, columns 1 and 2).

TABLE II

EFFECTS UPON PROPORTION OF SURVIVORS AND FREQUENCY OF VARIANT COLONIES OF TREATMENT FOR THREE HOURS AFTER PHOTOREACTIVATION

Expt. No.	Untreated photoreactivated control* (1)	Photoreactivated spores treated for 3 hr. at 37° C. with	
		Distilled water (2)	Iodoacetate (3)
(a) Proportion of survivors $\times 10^2$			
1	8.8	15.4	16.2
2	5.2	11.9	18.9
3	1.4	2.3	6.7
(b) Frequency of variants (%)			
1	33.2 ± 4.0	31.5 ± 2.6	31.8 ± 2.5
2	30.2 ± 1.7	34.6 ± 3.7	33.6 ± 3.0
3	34.1 ± 3.2	37.2 ± 2.5	34.6 ± 4.6

NOTE: *Irradiated spores were photoreactivated for 10 min. and then incubated at 37° C. in distilled water and with* 2×10^{-4} *M sodium iodoacetate (pH 7.2).*

* *Spores plated immediately after photoreactivation and dilution.*

Two possible mechanisms of action of iodoacetate were considered. These were stimulation of the rate of the reactions initiated by exposure to visible light, and inhibition of the loss of photoreversibility which occurs as the interval between ultraviolet and visible radiation is increased. The first possibility was tested in the following manner. A suspension of irradiated spores was incubated, without dilution, at 37° C. At various intervals 4-ml. samples were withdrawn and diluted with 1 ml. of either distilled water (control) or $10^{-3}M$ sodium iodoacetate (pH 7.2) (treated). The suspensions were then photoreactivated and plated. Only one suspension could be photoreactivated at one time, and samples treated with iodoacetate were photoreactivated 10 min. after the corresponding controls. The effects upon the proportions of survivors are illustrated in Table III.

The major observation was that when spores were incubated in distilled water for two hours or longer before the iodoacetate was added and the spores photoreactivated, the proportion of survivors was no greater than among control spores: and could even be less. Thus, it seemed probable that the major effect of treatment with iodoacetate was not stimulation of the rate of reactions initiated by photoreactivation, but rather inhibition of loss of photorevivability. This conclusion was substantiated by a study of the

TABLE III

EFFECT OF THE PRESENCE OF IODOACETATE DURING PHOTOREVERSAL UPON THE PROPORTION OF SURVIVORS AND THE FREQUENCY OF VARIANT COLONIES

Time of incubation in hr. prior to treatment with and without iodoacetate	Viable count/ml. × 10^8		Frequency of variants (%)	
	Without iodoacetate	With iodoacetate	Without iodoacetate	With iodoacetate
0	269	396	40.6 ± 3.4	34.6 ± 2.5
2	314	329	39.8 ± 2.9	40.7 ± 2.7
4	135	161	52.8 ± 4.5	50.9 ± 3.9
6	54	58	49.5 ± 2.2	50.0 ± 2.5
0	261	393	33.4 ± 2.9	39.2 ± 2.5
2	254	235	43.4 ± 3.1	50.0 ± 3.3
4	67	45	43.1 ± 2.3	42.7 ± 2.3
6	21	5.4	49.8 ± 3.5	38.9 ± 2.1

NOTE: *For details of the experiment see text.*

effects of varying the periods of treatments with iodoacetate and with distilled water upon the increases in survival caused by photoreactivation (Fig. 1). For all periods of treatment up to six and a half hours the survival among iodoacetate-treated spores was greater than among controls (curves *A* and *B*). During the same period, the proportion of survivors for non-photoreactivated spores also increased more if iodoacetate was present (curves *C* and *D*). However, even after six and a half hours the increase in survival due to photoreactivation was considerably greater for the iodoacetate-treated spores (Table IV: Expt. 1 corresponds to Fig. 1).

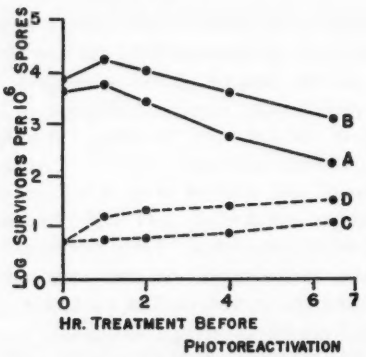


FIG. 1. Effect of postradiation treatment in distilled water and with iodoacetate upon the proportion of survivors obtained upon subsequent photoreactivation

Spores irradiated at a dose of 400 ergs/mm.^{2} were incubated at 37° C. either in distilled water of with 2×10^{-4} *M* sodium iodoacetate (pH 7.2). At intervals samples were plated prior and subsequent to photoreactivation for 10 min.

Curve *A*. Treated in distilled water and photoreactivated.
B. Treated in iodoacetate and photoreactivated.
C. Treated in distilled water.
D. Treated in iodoacetate.

TABLE IV

RATIOS OF PROPORTIONS OF SURVIVORS FROM IODOACETATE-TREATED AND CONTROL SUSPENSIONS BEFORE AND AFTER PHOTOREACTIVATION

Expt. No.	Proportion of survivors × 10^6				Ratios	
	Before photoreactivation		After photoreactivation			
	Control (*A*)	Treated (*B*)	Control (*C*)	Treated (*D*)	*B/A*	*D/C*
1	12.4	34.8	191	1330	2.8	7.0
2	8.3	15.8	170	690	1.9	4.1
3	4.7	5.9	128	236	1.3	1.8

NOTE: *Spores irradiated at a dose of 400 ergs/mm.² were treated for six and a half hours at 37° C. in distilled water (control) and with 2 × 10^{-4} sodium iodoacetate (pH 7.2) (treated). The suspensions were plated without and after photoreactivation for 10 min.*

When spores were photoreactivated in the presence of iodoacetate shortly after ultraviolet irradiation the proportion of survivors was consistently slightly higher than among the controls (Table III, Fig. 1, see also Table IV). Some of the increased survival could be accounted for by the 10 min. interval between the times of photoreactivation of treated and control samples, for there was a slight increase in the proportion of survivors during the early stages of incubation in distilled water (Fig. 1, curve *A*). Nevertheless, the difference in proportions of survivors between spores incubated with iodoacetate for one hour before photoreactivation and the corresponding controls was considerably greater than the expected differences between spores treated in water for periods varying by only 10 min. (Fig. 1).

The factors responsible for this transient slight effect of iodoacetate in causing increased survival have not been investigated. It seemed possible that iodoacetate did, in fact, stimulate the rate of the reactions initiated by exposure to visible light, in addition to inhibiting the loss of photorevivability. If these reactions were not normally completed during the 10 min. period of photoreactivation when the spores were photoreactivated immediately after ultraviolet radiation, iodoacetate would cause increased survival. However, the extent of photorevival decreased as the time of treatment in distilled water before photoreactivation was increased. Thus, as the period of treatment in distilled water was increased the time required for completion of the light-initiated reactions might decrease to less than 10 min. If that were so, stimulation of the rate of the reactions by iodoacetate would not necessarily cause increased survival.

As a first approach to determining whether iodoacetate did stimulate the rate of reactions initiated by photoreactivation, we performed the following experiment. Four-milliliter samples of an irradiated suspension were diluted with either 1 ml. of distilled water (control) or 1 ml. of $10^{-3}M$ sodium iodoacetate (pH 7.2) and photoreactivated for 10 min. and 20 min. Samples diluted with distilled water were also incubated for six hours and then

photoreactivated for 10 min. and 20 min. The results are given in Table V. For spores photoreactivated immediately after ultraviolet radiation, a 10 min. period of exposure to visible light was not adequate for the maximum possible increase in survival to take place. The rate at which the proportion of survivors increased was not a linear function of length of period of photoreactivation. Further, iodoacetate did stimulate the rate of increase in survival.

However, a 10 min. period of photoreactivation was also inadequate for maximal photorevival to occur with spores previously incubated in distilled water. Yet, for these spores the addition of iodoacetate prior to photoreactivation did not cause an increase in the extent of photorevival. Thus, the reactions postulated to be stimulated by iodoacetate either did not occur in spores previously incubated in distilled water, or else they were completed within a 10 min. period of exposure to visible light. Either possibility leads to the conclusion that a minimum of two distinct processes are involved in the processes of photorevival. We have, therefore, not pursued this line of investigation further.

When spores irradiated at a dose of 100 ergs/mm.^{2} were treated in distilled water and plated without photoreactivation the proportion of survivors rose and reached an approximately constant value after two to four hours treatment. Over the same period of time, the proportion of survivors among spores treated in distilled water and photoreactivated did not change significantly. For spores incubated with iodoacetate and plated without photoreactivation, the proportion of survivors rose slightly more rapidly than among the controls (4), but after photoreactivation the proportion of survivors did not differ significantly from that found among the controls. After treatment with iodoacetate for about four hours, the proportions of survivors obtained without and after photoreactivation progressively decreased as the period of treatment was increased.

Effects of Treatments with Iodoacetate and Light Upon the Frequency of Variant Colonies

As previously observed (4), postradiation treatment with iodoacetate caused a greater increase in the frequency of induced variant colonies than the control treatment in distilled water (Table I, columns 2 and 4). However, when the treated spores were photoreactivated, no significant difference was found between the frequencies of variants for iodoacetate-treated and for control spores.

When irradiated spores were immediately photoreactivated and then incubated for three hours, either with iodoacetate or in distilled water, there were no significant changes in the proportion of spores giving rise to variant colonies (Table II).

In contrast to the slow rate of increase in survival during photoreactivation, it was found that maximum photorestoration occurred during a 10 min. period of exposure to visible light (Table V).

TABLE V

EFFECT OF DURATION OF PHOTOREACTIVATION UPON THE PROPORTION OF SURVIVORS AND FREQUENCY OF VARIANT COLONIES

Expt. No.	Untreated control*	Spores photoreactivated without treatment				Spores photoreactivated in water after treatment in distilled water for 6 hr.	
		Photoreactivated in water		Photoreactivated in 2×10^{-4} *M* iodoacetate			
		10 min.	20 min.	10 min.	20 min.	10 min.	20 min.
(a) Proportion of survivors $\times 10^6$							
1	2.4	17,800	58,500	24,700	74,300	304	640
2	5.2	3,070	18,600	4,980	24,300	248	561
3	—	1,780	9,890	—	—	260	339
(b) Frequency of variants (%)							
1	40.5 ± 2.8	38.3 ± 3.2	33.5 ± 1.8	35.4 ± 2.7	32.7 ± 1.5	55.4 ± 2.5	46.3 ± 1.8
2	27.4 ± 1.9	35.6 ± 2.6	35.1 ± 3.4	39.8 ± 2.1	34.4 ± 2.9	49.2 ± 3.1	50.8 ± 2.0
3	—	33.4 ± 2.9	36.9 ± 4.4	—	—	50.8 ± 2.9	44.6 ± 2.2

NOTE: *Spores irradiated at a dose of 400 ergs/mm.² were photoreactivated as indicated before and after treatment in distilled water at 37° C.*
* *Suspension plated without treatment or photoreactivation immediately after ultraviolet radiation.*

When irradiated spores were incubated in a nutrient medium for varying periods of time and then photoreactivated, it was found that the photorestorability was lost sooner than the photorevivability (Newcombe and McGregor, unpublished experiments). Our data *suggest* that photorestorability is similarly lost more rapidly than photorevivability during the course of incubation in distilled water. Photorevival was clearly observed after periods of incubation of up to six and a half hours (Fig. 1), whereas photorestoration was not observed after treatment for four hours (Table VI).

With spores treated in iodoacetate for any period and then photoreactivated, the frequency of induced variant colonies was usually insignificantly different from that observed with the corresponding control (Table VI). However, two factors complicate the interpretation of these results. First, during the course of treatment with iodoacetate, the frequency of variant colonies observed for non-photoreactivated spores rapidly rose to values which did not differ significantly from those obtained when the same spores were photoreactivated. Second, the dose of radiation employed (400 ergs/mm.^{2}) was deliberately selected as one slightly in excess of that giving the maximum frequency of variants on the "dose–mutation curve" (4). Thus, an appropriate reduction in effective radiation dose could, by coincidence, mask an effect of iodoacetate treatment upon the extent of change in frequency of induced variant colonies.

These complications can be avoided by using a radiation dose of 100 ergs/mm.^{2}, because treatment of spores irradiated at this dose (either in iodoacetate or in distilled water) does not cause any significant change in the frequency of induced variant colonies (3, 4). Also, the dose is well below that corresponding to the peak of the "dose-mutation curve" (4). Experiments were therefore performed with spores irradiated at this dose. Again we observed no significant effects of iodoacetate-treatment upon either the extent of photorestoration or the rate of loss of photorestorability (Table VII). As noted above, treatment with iodoacetate for more than four hours proved somewhat toxic to spores irradiated at this lower dose, but it seems most unlikely that the toxic effect of prolonged treatment exactly balanced an effect upon the processes involved in the phenomenon of photorestoration.

Loss of Response to Treatment with Iodoacetate

For comparison with the studies of the losses of photorevivability and photorestorability, we have studied the rate of loss of ability to respond to treatment with iodoacetate. A suspension of irradiated spores was incubated in distilled water at 37° C. At intervals 4-ml. samples were withdrawn, diluted with 1 ml. of either distilled water of $10^{-3}M$ sodium iodoacetate (pH 7.2), re-incubated for four hours, and plated. When iodoacetate was added after treatment in distilled water for two to four hours the subsequent increases in proportion of survivors and the variant frequency did not differ significantly from those observed with the control spores (Table VIII). Thus, the ability of irradiated spores to respond to treatment with iodoacetate

TABLE VI

EFFECTS OF PRETREATMENT IN DISTILLED WATER AND WITH IODOACETATE UPON THE FREQUENCY OF VARIANT COLONIES BEFORE AND AFTER PHOTOREACTIVATION

Expt. No.	Treatment in	Photo-reactivated	Frequencies of variants (%) after: 0 hr.	1 hr.	2 hr.	4 hr.	6½ hr.
	Water	−	27.1 ± 2.2	24.1 ± 1.9	29.2 ± 1.9	32.7 ± 2.5	42.4 ± 4.6
1		+	37.9 ± 2.4	40.2 ± 2.1	46.5 ± 3.1	48.6 ± 2.2	51.0 ± 3.8
	Iodoacetate	−	28.4 ± 2.0	46.4 ± 4.0	48.2 ± 3.4	57.1 ± 3.2	52.9 ± 2.8
		+	32.4 ± 1.8	41.6 ± 3.8	45.4 ± 4.8	46.6 ± 2.6	52.8 ± 1.4
	Water	−	31.1 ± 1.9	34.2 ± 1.7	35.9 ± 1.9	46.6 ± 4.2	54.8 ± 3.5
2		+	44.8 ± 1.8	45.6 ± 3.6	47.6 ± 3.2	53.6 ± 3.0	51.2 ± 2.6
	Iodoacetate	−	35.9 ± 3.1	53.1 ± 2.6	58.9 ± 2.5	49.6 ± 1.7	55.8 ± 3.0
		+	38.5 ± 0.9	51.1 ± 5.1	45.2 ± 1.7	53.9 ± 2.6	45.6 ± 4.4
	Water	−	36.1 ± 3.5	30.6 ± 3.3	37.3 ± 3.0	42.3 ± 3.0	45.9 ± 2.4
3		+	(62.5 ± 12.1)	45.4 ± 4.0	46.2 ± 3.3	51.9 ± 2.4	51.3 ± 4.5
	Iodoacetate	−	38.6 ± 3.4	53.9 ± 3.2	50.3 ± 2.3	53.9 ± 2.2	54.5 ± 2.1
		+	(61.2 ± 6.2)	56.5 ± 4.2	56.2 ± 4.3	51.9 ± 2.0	54.9 ± 1.0

NOTE: *Irradiated spores were treated at 37° C. in distilled water and with 2×10^{-4} M sodium iodoacetate (pH 7.2). At various intervals samples were plated without and after photoreactivation for 10 min.*

TABLE VII

EFFECTS OF PRETREATMENT IN DISTILLED WATER AND WITH IODOACETATE UPON THE FREQUENCY OF VARIANT COLONIES BEFORE AND AFTER PHOTOREACTIVATION OF SPORES IRRADIATED AT A DOSE OF 100 ERGS/MM.^{2}

Expt. No.	Treated in	Photo-reactivated	Frequencies of variants (%) after:				
			0 hr.	1 hr.	2 hr.	4 hr.	6½ hr.
1	Water	−	13.6 ± 1.3	13.7 ± 1.2	13.6 ± 3.5	16.3 ± 1.3	11.6 ± 1.0
		+	8.0 ± 0.9	11.8 ± 1.1	11.5 ± 3.1	11.4 ± 3.6	12.9 ± 1.1
	Iodoacetate	−	13.8 ± 1.3	12.2 ± 1.1	13.7 ± 1.3	13.0 ± 1.3	15.3 ± 1.3
		+	8.2 ± 0.9	11.4 ± 3.2	8.1 ± 2.4	11.0 ± 1.1	15.5 ± 1.4
2	Water	−	11.5 ± 1.2	15.2 ± 3.5	12.1 ± 1.1	13.6 ± 3.0	18.8 ± 3.5
		+	6.9 ± 0.7	7.3 ± 2.3	7.3 ± 2.2	9.7 ± 2.6	16.7 ± 3.4
	Iodoacetate	−	10.2 ± 1.0	13.3 ± 1.1	12.5 ± 3.2	12.0 ± 1.2	13.7 ± 1.3
		+	8.3 ± 2.4	12.0 ± 2.8	8.4 ± 0.8	9.9 ± 3.0	12.5 ± 3.2

NOTE: *Irradiated spores were treated at 37° C. in distilled water and with 2×10^{-4} M sodium iodoacetate (pH 7.2). At various intervals samples were plated without and after photoreactivation for 10 min.*

TABLE VIII

EFFECT OF PRIOR TREATMENT IN DISTILLED WATER UPON THE RESPONSE TO FURTHER POSTRADIATION TREATMENT WITH IODOACETATE

Expt. No.	——	Proportion of survivors × 10^7 after:			Frequency of variants (%) after:		
		0 hr.	2 hr.	4 hr.	0 hr.	2 hr.	4 hr.
1	Plated without further treatment	39	65	98	30.8 ± 2.2	31.3 ± 1.7	42.0 ± 4.7
	Treated with distilled water for 4 hr.	71	92	120	39.6 ± 1.7	55.2 ± 4.9	51.5 ± 4.3
	Treated with iodoacetate for 4 hr.	261	160	116	57.8 ± 2.9	57.0 ± 3.7	51.8 ± 4.3
2	Plated without further treatment	96	161	170	27.1 ± 3.6	31.6 ± 3.7	32.0 ± 2.8
	Treated with distilled water for 4 hr.	151	156	170	34.6 ± 3.1	31.6 ± 3.3	35.4 ± 3.1
	Treated with iodoacetate for 4 hr.	242	166	174	49.1 ± 2.6	41.4 ± 3.0	38.1 ± 2.9

NOTE: *For details of experiment see text.*

was lost more rapidly during the course of treatment in distilled water than either the photorevivability (Fig. 1) or the photorestorability (Tables VI and VII).

Discussion

In this study we have made no attempt to elucidate the mechanism(s) responsible for the observed effects of photoreactivation. Rather, we have been concerned with the manner in which the effects of a standard period of exposure to visible light could be modified by treatments applied between the ultraviolet and visible irradiations. Nevertheless, one feature of the results obtained merits special attention, namely, that the mechanism of photorevival differs in very many respects from that of photorestoration. Of the many points of dissimilarity revealed in this survey perhaps those of greatest significance are the inhibition of loss of photorevivability by iodoacetate and the absence of effects of this agent upon the course of loss of photorestorability. The information currently available does not permit any detailed interpretation of the differences in the mechanisms of the processes of photorevival and of photorestoration, but does suggest an approach of potentially greater value than the "dose-reduction" concept. Indeed, it seems probable that similar (but more extensive) studies with iodoacetate and other agents known to affect the process(es) of photorevival (1, 2) will give some indication of the mechanism by which exposure to visible light results in reversal of the lethal effects of ultraviolet radiation.

The results of the present study further emphasize that the effects of postradiation treatments with iodoacetate are probably due to actions upon the spore metabolism. To account for the results on the basis of a simple chemical model would require both an increase in the number of hypothetical radiation-produced "poisons" previously postulated (3, 4, 5) and an additional classification based upon their rates of interaction with cell constituents during the interval between the ultraviolet and visible radiations.

On the other hand, as far as we are aware, photoreactivation and loss of photoreversibility have not been found to occur other than under conditions of active cell metabolism. Thus, the effects of visible light upon irradiated spores of *Streptomyces* T12 and the loss of photoreversibility which occurs during postradiation treatment in distilled water may be considered as evidence that the spores do contain appropriate utilizable metabolic reserves. Further, and more important, the inhibition of loss of photorevivability by iodoacetate (Fig. 1) may be taken as evidence that metabolism of those reserves is, in fact, inhibited by the concentrations of iodoacetate used in earlier experiments (3, 4).

As has been noted elsewhere (3), there is no reason to suppose that the metabolic disturbance resulting from ultraviolet radiation is either quantitatively, or even qualitatively, the same for all doses of radiation. Therefore, it is not surprising that postradiation treatment with iodoacetate of spores irradiated at doses of 100 ergs/mm.^{2} for periods of four hours or longer results

in a decrease in the proportion of survivors, whereas for spores irradiated at doses of 400 ergs/mm.^{2} similar treatment results in a continued stimulation of the rate of increase in proportion of survivors (4). Similarly, any disturbance of the course of cell metabolism which could determine whether a "potentially-variant" (provariant) spore would yield a variant or non-variant colony must presumably be a subtle change in comparison with the gross disturbances likely to influence spore survival. Thus, it is not surprising that treatment with a single agent (iodoacetate) may affect survival under a variety of postradiation conditions, but may affect the variant frequency only under selected conditions. In particular, it is not surprising that iodoacetate may inhibit loss of photorevivability but not loss of photorestorability.

References

1. BERGER, H., HAAS, F. L., WYSS, O., and STONE, W. S. Effect of sodium azide on radiation damage and photoreactivation. J. Bacteriol. 65 : 538-543. 1953.
2. JACOBS, L. Private communication.
3. WAINWRIGHT, S. D. and NEVILL, A. Modification of the biological effects of ultraviolet irradiation by post irradiation treatment with iodoacetate and peptone. J. Gen. Microbiol. 12 : 1-12. 1955.
4. WAINWRIGHT, S. D. and NEVILL, A. Induction of provariants as a function of dose of ultraviolet radiation. J. Gen. Microbiol. 12 : 13-24. 1955.
5. WAINWRIGHT, S. D. and NEVILL, A. Some effects of postirradiation treatment with metabolic inhibitors and nutrients upon ultraviolet irradiated spores of *Streptomyces* T12. Can. J. Microbiol. 1 : 416-427. 1955.

ON SOME PARASITES OF PARASITIC PROTOZOA

I. *SPHAERITA HOARI* SP. N.—A CHYTRID PARASITIZING *EREMOPLASTRON BOVIS*[1]

By G. Lubinsky

Abstract

Sphaerita hoari sp. n.—a chytrid parasitizing *Eremoplastron bovis*—is described with special attention given to its vegetative structures. *Sphaerita*-like parasites of nine species of other ciliates of the rumen of domestic ruminants are reported. It is shown that at least some of these parasites are chytrid fungi.

The study of parasites[2] of parasitic protozoa is both of considerable theoretical and of potential practical interest. One of the most common parasites of intestinal protozoa—pathogenic as well as nonpathogenic—is *Sphaerita*, a lower fungus belonging to the family Olpidiaceae (Chytridiales). The genus *Sphaerita*, with the genotype *Sphaerita endogena*, was established by Dangeard in 1886 (6, 7) for a chytrid parasitizing free-living rhizopods—*Nuclearia simplex* and *Heterophrys dispersa*. Later Dangeard (8) studied another species of *Sphaerita* parasitizing *Euglena*—a species subsequently named by Chatton and Brodsky (3) *Sphaerita dangeardi*. As this last species was more adequately studied than *S. endogena*, Dangeard in 1933 has proposed designating it as the type species of the genus (Sparrow, 25).

In 1909 Chatton and Brodsky found *S. endogena* in a free-living amoeba, which they tentatively identified as *Amoeba limax* Dujardin. They have described both the thallus and the formation of sporangia of this parasite. There can be little doubt that the species described by them is indeed a chytrid fungus.

A few years after the publication of Chatton and Brodsky's paper, *Sphaerita*-like organisms were found in all species of intestinal amoebae of man (10, 12, 13, 21, 26, 27). They were also shown to be common in intestinal protozoa of rodents as, for example, in *Entamoeba muris* (16, 29), *E. citelli* (1, 22), *E. babaci* (20,) in intestinal amoebae of monkeys, cattle, and other mammals, as well as in amoebae of lower vertebrates. *Sphaerita*-like parasites were also found in flagellates of termites (17, 18) and in intestinal trichomonads of rodents (5, 19). A few cases of their presence in free-living ciliates are on record (2, 4). Sassuchin (22, 23, 24) found them in *Nyctotherus ovalis* from the intestine of a cockroach. Dogiel (11), Jiroveč (15), Das Gupta (9), and Winogradowa (30) have described *Sphaerita*-like organisms in endozoic ciliates of the family Ophryoscolecidae from the rumen of ruminants.

[1] *Manuscript received February 25, 1955.*
Contribution from the Institute of Parasitology, McGill University, Macdonald College, Quebec, Canada, with financial assistance from the National Research Council of Canada.

[2] *In this paper the term "parasite" is used in the sense of Braun (1891) to designate an organism inhabiting another organism independently of its pathogenicity or usefulness to the host. The term is thus synonymous with de Bary's term "symbiote".*

In most of these observations only the presence of sporangia-like bodies was recorded, but the morphology of vegetative structures of *Sphaerita*-like organisms was not studied. Sparrow (25) in his monograph on aquatic Phycomycetes has not discussed the *Sphaerita* of endozoic protozoa because of the inadequacy of the morphological data available. Kirby (18), discussing the systematic position of the symbiote Gh 5—a *Sphaerita*-like parasite of *Gigantomonas herculea*—wrote:

"In many organisms (described as *Sphaerita* by protozoologists (Autor)) we do not even know that a multinucleate thallus is formed; the sphaerules that are seen may perhaps be isolated at all times. Studies on fixed and stained material should, if complete, be more adequate morphologically than those based only on living material; but none of the accounts of so-called *Sphaerita* in endozoic protozoa have provided indisputable evidence of the presence of the features necessary for placement in that genus."

The words of Kirby, although written in 1946, perfectly summarize our present-day knowledge of *Sphaerita*-like parasites of endozoic protozoa and stress the need for their critical re-examination.

The faunules of the rumen of ruminants at my disposal contain numerous ciliates parasitized by *Sphaerita*-like organisms and are thus favorable for detailed morphological studies of these peculiar hyperparasites.

Material and Methods

The material—rumen contents of sheep, Jamnapari goats, Indian cattle *(Bos indicus)*, and water buffaloes *(Bubalus bŭbalus)*—was collected by me at the butchery in Rawalpindi-Cantt, West Pakistan, in 1951–1953. It was fixed with a 4% formaldehyde (10% formalin). Total mounts were made using a simplification of Westphal's (28) method: fixed gastric contents were twice washed with distilled water by centrifuging and the washed sediment mixed with twice its volume of human serum. Drops of the mixture were placed on slides and spread to the size of circles of about 18mm. in diameter. The slides were put in a moist chamber with formaldehyde vapor for 24 hr. The coagulated films were handled as histological sections, and were stained with Hoare's modification (14) of Mayer's acid haemalum. A few slides were counter-stained with orange G.

Results

In this material *Sphaerita*-like organisms were found in 10 species of endobiotic ciliates of the rumen. In Table I are summarized data on their prevalence and distribution in the infected ciliates and the mammalian hosts of the latter. Usually only a small percentage of the ciliates were parasitized, the only heavily infected species being *Eremoplastron bovis* from one Jamnapari goat; 19% of these ciliates contained sporangia of *Sphaerita*-like organisms. Thus this species provided suitable material for the study of the life cycle of the *Sphaerita*-like organism parasitizing it.

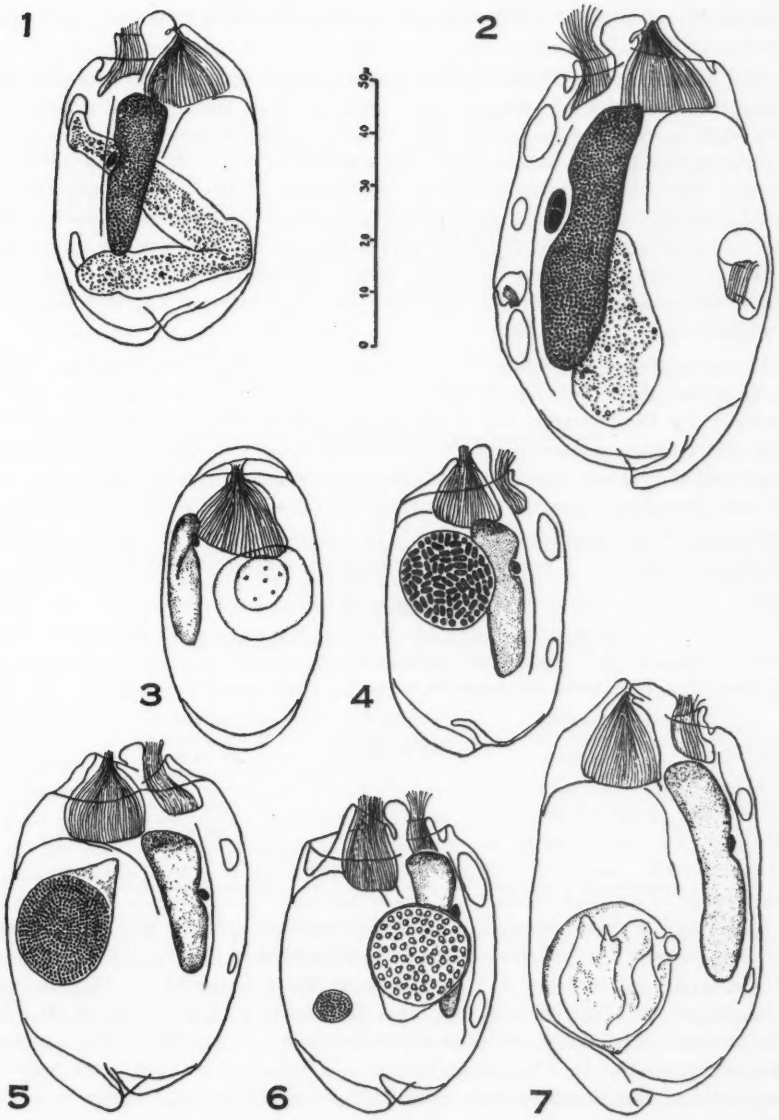
TABLE I

Sphaerita-LIKE ORGANISMS IN CILIATES OF THE RUMEN OF DOMESTIC RUMINANTS IN RAWALPINDI, WEST PUNJAB

Host of *Sphaerita*-like organisms	Host of ciliate	Percentage of ciliates containing sporangia
Ophryoscolecidae:		
1. *Entodinium dubardi* Buiss 1923	Sheep	<1
2. *E. ovinum* Dogiel 1927	Goat	<1
3. *E. longinucleatum* Dogiel 1925	Goat	<1
4. *E. loboso-spinosum* Dogiel 1925	Sheep	<1
5. *E. bicarinatum* Cunha 1914	Buffalo	<1
6. *E. tricostatum* Kofoid & MacLennan 1930	Buffalo	<1
7. *E. indicum* Kofoid & MacLennan 1930	Buffalo	2
8. *Eremoplastron bovis* (Dogiel 1927)	Goat	19
9. *Epidinium caudatum* (Fiorentini 1889)	Goat	<1
Isotrichidae:		
10. *Isotricha intestinalis* Stein 1858	Sheep	<1

The earliest stage of development of the fungus found in *E. bovis* is a naked bandshaped plasmodial thallus with slightly irregular outlines reminiscent of those of a creeping amoeba of the "limax" type. The plasmodium contains several small spherical nuclei about 0.5μ in diameter, situated in the granular endoplasm. The ectoplasm is clear, homogenous, and much less basophilic than the endoplasm of the host. Although usually irregularly ellipsoidal, the plasmodium may attain considerable length, as shown in Fig. 1. We were however unable to decide whether in this case we had to do with one plasmodium bent near the ventral surface of the host, or with two bandshaped plasmodia with superimposed ends. The plasmodial thallus is mostly situated in the endoplasm of the ciliate near its macronucleus, but may also extend into the ectoplasm of the host. It is remarkable that ciliates, which contain plasmodia of considerable size, may divide (Fig. 2). It is probable that, in the course of division, the thallus is sometimes cut by the division furrow, the two parts of the parasite being thus distributed between the two daughter ciliates.

The formation of the sporangium starts with the contraction of the plasmodium which assumes a spherical or subspherical shape (Fig. 3). The contrast between the finely granular dark endoplasm and the nearly homogenous light ectoplasm increases. At this stage the number of nuclei varies from four to about twenty depending on the size of the sporangium formed. The spherical nuclei, about 0.5μ in diameter, are widely spaced (Fig. 3) in the endoplasm. Soon intensive multiplication of nuclei sets in, all nuclei dividing simultaneously (Fig. 4). Because of the minute size of the nuclei, it is difficult to see the details of this process. Dividing nuclei assume an elongate-cylindrical form with semispherical ends and measure at that stage about 1μ by 2μ. Soon the whole sporangium becomes filled with



FIGS. 1 to 7. *Sphaerita hoari* sp. n. in *Eremoplastron bovis*. Fig. 1. Two band-shaped thalli (or one long?). Fig 2. Dividing specimen of *E. bovis* with a thallus near its macronucleus. FIG. 3. A young sporangium. FIG. 4. Simultaneous nuclear divisions in a ripening sporangium. FIG. 5. Microsporous and FIG. 6. Macrosporous sporangia with discharge papillae. FIG. 7. Sporangium after discharge of zoospores.

nuclei, the width of spaces between them being about half their diameter (Fig. 5). It is difficult to decide whether the spores are delimited simultaneously or progressively.

The spore-ball is surrounded by a thin inner wall of the sporangium, separated from the exosporium by a layer of clear homogenous substance. The wall of the exosporium is very thin in young sporangia but becomes thicker as the sporangium matures, and is seen as a refractile outer membrane at the time of formation of discharge papilla. The inconspicuous outer membrane of young sporangia and the homogeneity of the substance filling the space between it and the inner wall make one believe that the spore-ball with the inner membrane forms the whole sporangium, the space between the inconspicuous exosporium and the inner membrane being mistaken for a vacuole formed by the host.

In our material two kinds of sporangia were present: microsporous, with spore nuclei about 0.5μ in diameter (Fig. 5), and macrosporous, with nuclei about 1.5μ in diameter and a refractile globule about 0.5μ in diameter adjacent to each nucleus (Fig. 6). The first type was much more common than the second, of which only a few specimens were seen. The infected ciliates contained mostly one sporangium, specimens with two or three sporangia being comparatively rare. Numbers of sporangia found in each of 200 specimens of *E. bovis* with sporangia are summarized in Table II.

TABLE II

NUMBER OF SPORANGIA IN 200 *Eremoplastron bovis* SPECIMENS CONTAINING SPORANGIA

Number of sporangia in one ciliate	Number of *E. bovis* bearing sporangia	Percentage of *E. bovis* bearing sporangia
1	157	78.5
2	39	19.5
3	4	2

Diameters of 100 sporangia from hosts containing only one sporangium were measured. The variation curve is represented in Fig. 8. The diameters of sporangia varied from 4.5 to 21μ with the average of $12.77 \pm 0.37\mu$, a standard deviation of $\pm 3.68\mu$, and the quotient of variation of 20.9%. The variability of the size of sporangia was thus considerable. The ripeness of sporangia is heralded by the formation of a broad conical discharge papilla, nearly triangular in side view, with its basis coalescing tangentially with the walls of the spherical or subspherical sporangium, which thus becomes pear-shaped. The contents of the discharge papilla are clear and appear nearly colorless even in overstained specimens. The length of the papilla never exceeds half the diameter of the sporangium. The discharge pore is formed by deliquescence of the extramatrical tip of the papilla. After the discharge

of zoospores the membrane, which prior to discharge surrounded the spore mass, is still visible in the empty sporangium (Fig. 7). Unfortunately in our material we have not found sporangia fixed at the time of discharge. The flagellation of zoospores was therefore not studied.

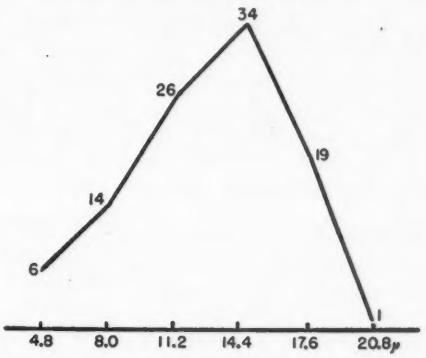


FIG. 8. Dimensions of single microsporous sporangia of *S. hoari*. ($M = 12.77 \pm 0.37\ \mu$; $\sigma = \pm 3.68\mu$; $n = 100$.)

The fungus appears to do little harm to its host. We have seen many ciliates dividing despite the presence of several thalli in their endoplasm (Fig. 2). However, *Eremoplastron* containing several ripe sporangia often degenerate: the chromatin granules of the macronucleus coalesce and form lumps, the cytoplasm becomes edematous, and finally the ciliate disintegrates. However, ciliates with empty sporangia are often seen undergoing divisions.

Discussion

The fungus described above certainly fits into the range of microorganisms which are designated by protozoologists as "*Sphaerita*". To it, as well as to other *Sphaerita*-like organisms parasitizing endobiotic protozoa, are fully applicable the words of Kirby quoted in the Introduction. However, the comparative abundance of material at our disposal enabled us to investigate the life cycle of our fungus more completely than is possible when only a small percentage of a host species is infected, and thus to answer some of the questions put forward by Kirby.

Our material, although not adequate to elucidate the full life cycle of the fungus, is still sufficient to prove that we have not to do with assemblages of spherules which "have been isolated at all times", but with sporangia of a lower fungus. The presence of a holocarpic monocentric endobiotic thallus, the structure of the single sporangium produced, and the presence of refractile globules in the spores of at least the macrosporous sporangium compel us to place our fungus tentatively in the family Olpidiaceae (Chytridiales). It is obvious that this fungus does not belong to the genus *Olpidimorpha*, *Nucleophaga*, or *Pringsheimiella*. Neither can it be placed in the genus

Rozella or *Pleotrachelus*, because it does not fill the host cells and parasitizes a ciliate. The remaining two genera of Olpidiaceae—*Olpidium* and *Sphaerita*—both contain species parasitizing protozoa. We can exclude *Olpidium* because of the absence of a discharge tube in our species. This character can not be explained by scarcity of material as we have examined over 500 specimens of our fungus including many empty sporangia. Its characteristics closely fit those of the genus *Sphaerita*. Only one important point does not coincide with the characters of this genus: the presence in our fungus of a naked thallus. It has to be noted, however, that the vegetative structures in the genus *Sphaerita* are not sufficiently known. It seems to be advisable therefore to place our species in the genus *Sphaerita*, pending a further study of the vegetative structures of its type species.

Our fungus differs from both *S. dangeardi* and *S. endogena* in the size of the spores, which have a diameter of 1.5–2.0μ in the above two species but only 0.7μ (with a nucleus of 0.5μ) in the microsporous sporangia of our species. The spores produced in the rare macrosporous sporangia are, on the other hand, larger than those of *S. dangeardi* and *S. endogena*. Peculiar for our fungus also is its habitat; it parasitizes a ciliate *(Eremoplastron bovis)*, whereas *S. dangeardi* lives in free-living rhizopods, and *S. endogena* in *Euglena*.

Only three species of the genus *Sphaerita* have been described as parasitizing endozoic ciliates of vertebrates. The descriptions of these parasites are confined to a few morphological features of sporangia. In 1929, Dogiel described *Sphaerita diplodiniorum* from *Diplodinium costatum* and *Ostracodinium gracile*, found by him in African antelopes—*Bubalis cokei* and *Raphiceros* sp. respectively. In his description (11) he writes: "The parasites are situated in the endoplasm of the infusorian and are always numerous. They form regular morula-shaped spore-balls with radiary arranged spores. Up to 30–40 spore-balls can be found in the endoplasma-sack of a single infusorian." The accompanying picture (Fig. 5 of his paper) shows morula-shaped assemblages of spherical or ovoidal bodies without any membrane surrounding the assemblage. As far as can be judged from this picture, each spore-ball contains a few dozen "spores". The statement of Kirby (17, p. 1050) that "Dogiel (1929) found only from 30 to 40 spores in the "spore-ball" of *S. diplodiniorum*" is probably a misunderstanding of the above-quoted statement of Dogiel concerning the number of "spore-balls" in one ciliate. Although Dogiel's description of *S. diplodiniorum* is not complete and does not provide the characteristics necessary for the placement of this organism in the class Phycomycetes, the difference between our fungus and Dogiel's *S. diplodiniorum* as to the size and number of spores is obvious.

In 1933 Jirovec described two species of *Sphaerita* in Ophryoscolecidae: *S. entodinii minor* and *S. entodinii major*. He does not explain why trinominals were used. In a few lines he describes "ripening cysts of *Sphaerita*" of the first species which he has found in *Entodinium simplex* (?) (interrogation sign by Jiroveč). The species is probably *E. contractum* Kofoid and

Christensen 1934 (see Jirovec (1933), Fig. 5*a* and *b*). The cysts are 5–10μ in diameter and filled with darkstained "very small bodies".

The second species — *S. entodinii major* — was found by Jirovec in *E. longinucleatum*. Its description is limited to the words "Im Plasma kamen 10–15μ grosse, helle Kugeln vor mit grossen dunkelgefärbten Sporen (1–1½μ in Durchmesser)". The lack of description of vegetative structures and the inadequacy of the description of what may have been sporangia does not justify the placement of the above organisms in the genus *Sphaerita* and does not give any evidence of their being fungi. Thus *S. entodinii minor* and *S. entodinii major* can not be regarded as valid species.

No other species of *Sphaerita* have been described from endozoic ciliates of mammals, though Winogradowa (30) has reported in *Entodinium* sp. the presence of spherical or ovoidal collections of small round and ovoidal bodies not over 1μ in diameter and without any membrane surrounding the collection. Das Gupta (9) recorded the presence of "Sphoeritta" in *Entodinium caudatum* from Indian goats.

From the obvious differences between our fungus and the known species of *Sphaerita* and *Olpidium*, both in structure and in host, we consider it to be a new species and name it *Sphaerita hoari* in honor of Dr. C. A. Hoare, F.R.S., protozoologist to the Wellcome Research Institution.

Sphaerita hoari sp. n.

Thallus holocarpic, endobiotic, naked, plasmodial with granular endoplasm and hyaline ectoplasm, ellipsoidal or band-shaped, with numerous nuclei 0.5μ in diameter. Sporangia spherical or subspherical 12.8 (4–21)μ in diameter, producing a broad discharge papilla nearly triangular in side view, without operculum. Microsporous sporangia of average size contain several thousands of spores with nuclei about 0.5μ in diameter. The rare macrosporous sporangia contain a few hundred spores with nuclei 1.5μ in diameter each with an adjacent refractile globule 0.5μ in diameter. Flagellation of spores not known. Resting spores not seen.

Found parasitizing *Eremoplastron bovis* Dogiel 1925 (Ophryoscolecidae) from the rumen of *Capra hircus* L. in Rawalpindi, West Pakistan, in July 1951.

Type specimen in the Institute of Parasitology, McGill University, Macdonald College, Quebec.

Sphaerita hoari sp. n.

Thallus holocarpicus, endobioticus, nudus, plasmodialis, ellipsoideus aut taeniaformis, cum ectoplasma hyalina et endoplasma granulosa. Nuclei numerosi 0.5μ diametro. Sporangiae sphaericae aut subsphaericae 12.8 (4–21)μ diametro, papillae quae zoospores emittunt singulae, breves et latae, in aspecto laterali trianguliformes; nullo operculo.

Sporangiae microsporiae mediocris magnitudinis nonnulla milia sporarum continent, qui nucleos 0.5μ diametro habent. Aliquae rarae sporangiae macrosporiae nonnulla centum sporas continent qui nucleos 1.5μ diametro habent quorum ad unum quemque globulus refractivus adiectus est. Flagellatio zoosporarum ignorata est. Sporae perdormientes non visae sunt.

Reperta parasitica in *Eremoplastron bovis* Dogiel 1925 (Ophryoscolecidae) ex ruminis *Capra hircus* L. prope Rawalpindi, W. Pakistan, mense Juli, 1951.

It is possible that the study of the flagellation of zoospores in this species, as well as further studies of vegetative structures of the type species of the genus *Sphaerita*, may result in the segregation of *S. hoari* into a genus of its own.

Several species of Ophryoscolecidae in my material, other than *E. bovis*, were found to contain *Sphaerita*-like parasites (see Table I). Unfortunately only a very low percentage of host species were infected in all cases.

All sporangia-like structures seen in these Ophryoscolecidae possessed a membrane surrounding the collection of spherules. Some collections, e.g. those found in *Entodinium bicarinatum* (Fig. 9) and in *Epidinium caudatum*, closely resembled sporangia of *S. hoari*. In one faunule from the rumen of a Jamnapari goat, a few specimens of *Entodinium ovinum* were found to contain parasites similar to sporangia of *Sphaerita*. One specimen of *E. ovinum* from the same faunule contained a peculiar naked, lobed plasmodium with numerous vesicular nuclei about 2μ in diameter, each with one adjacent refractile globule (Fig. 10). It is probable that this structure, as well as sporangia-like inclusions in the above *Entodinium*, belongs to a chytrid fungus.

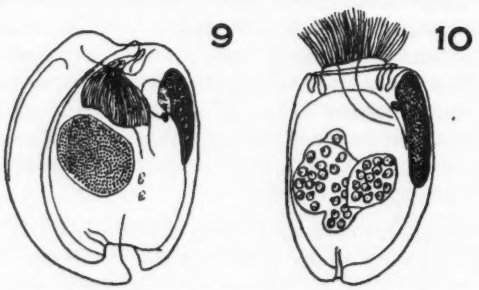


FIG. 9. *Sphaerita*-like parasite in *Entodinium bicarinatum* Da Cunha 1914.
FIG. 10. Plasmodium of a chytrid fungus (?) in *E. ovinum*.

Are *Sphaerita*-like parasites of intestinal amoebae and flagellates simple assemblages of spherical microorganisms, as Kirby surmised, or sporangia of chytrids? No sweeping answer can be given to this question. One feature of these parasites has to be pointed out, however: their nuclei are usually described as being highly refringent—a physical property which is at variance with properties of any nuclei known and is reminiscent of the refractile globules of chytrids. It is probable, therefore, that some of these parasites

are chytrid fungi. Some others, e.g. *Sphaerita endamoebae* Becker 1926 and *Sphaerita wenrichi* Crouch 1933, are collections of solitary microorganisms multiplying in a vacuole formed by the host, as pointed out by Kirby and Honigberg (1949). Still others, e.g. the "sphaerita-like" parasite depicted by Jirovec (1933, Fig. 5*d*), are food particles—in this case, spherical droplets of semidigested nuclei of engulfed ciliates. The literature on the "Sphaerita" of intestinal amoebae and flagellates is confusing, sometimes limited to a few lines in papers dealing with other questions and scattered in hundreds of biological and medical journals. The answer to the above question requires revision of this literature and further study of the material available.

References

1. Becker, E. R. *Endamoeba citelli* sp. nov. from the striped ground squirrel *Citellus tridecemlineatus*, and the life history of its parasite *Sphaerita endamoebae* sp. nov. Biol. Bull. 50 : 444-454. 1926.
2. Cejp, K. *Sphaerita*, parasit Paramecii. Spisy Prirodovedeckou Fak. Univ. Karlovy, Praha, 141 : 3-7. 1935.
3. Chatton, E. and Brodsky, A. Le parasitisme d'une Chytridinée du genre *Sphaerita* Dangeard chez *Amoeba limax* Dujardin. Arch. Protistenk. 17 : 1-18. 1909.
4. Collin, B. Étude monographique sur les Acinetiens. II. Morphologie, physiologie, systématique. Arch. zool. exptl. et gén. 51 : 1-457. 1912.
5. Crouch, H. B. Four new species of *Trichomonas* from the woodchuck (*Marmota monax* Linn.). J. Parasitol. 19 : 293-301. 1933.
6. Dangeard, P. A. Sur un nouveau genre de Chytridinées parasite des Rhizopodes et des Flagellates. Bull. soc. botan. France, 33 : 240-241. 1886.
7. Dangeard, P. A. Recherches sur les organismes inférieurs. Ann. sci. nat. Botan. VII. 4 : 241-341. 1886.
8. Dangeard, P. A. Mémoire sur les Chytridinées. Le Botaniste, 1 : 39-74. 1889.
9. Das Gupta, M. Preliminary observations on the Protozoan fauna of the rumen of the Indian goat, *Capra hircus* Linn. Arch. Protistenk. 85 : 153-172. 1935.
10. Dobell, C. The amoebae living in man. John Bale, Sons and Danielsson Ltd., London. 1919.
11. Dogiel, V. Biologische Notizen über Darminfusorien der Huftiere. Arch. russes protistol. 8 : 257-262. 1929.
12. Epstein, H. On parasitic infections of intestinal amoebae (in Russian). Arch. russes protistol. 1 : 46-81. 1922.
13. Epstein, G. V. Pathogenic protozoa, spirochaetes and fungi. Moskva (textbook). 1931.
14. Hoare, C. A. Medical protozoölogy. Baillière, Tindall & Cox, Ltd., London. 1949.
15. Jiroveč, D. Beobachtungen über die Fauna des Rinderpansens. Z. wiss. Biol. Abt. F. Z. Parasitenk. 5 : 584-591. 1933.
16. Kessel, J. E. The distinguishing characteristics of the parasitic amoebae of culture rats and mice. Univ. Calif. Publs. Zoöl. 20 : 489-544. 1924.
17. Kirby, H., Jr. Organisms living on and in Protozoa. Protozoa in Biological Research. Columbia University Press, New York. 1941. pp. 1009-1113.
18. Kirby, H., Jr. *Gigantomonas herculea* Dogiel, a polymastigote flagellate with flagellated and ameboid phases of development. Univ. Calif. Publs. Zoöl. 53 : 163-226. 1946.
19. Kirby, H. and Honigberg, B. Flagellates of the caecum of ground squirrels. Univ. Calif. Publs. Zoöl. 53 : 315-366. 1949.
20. Li-Iuan-Po. *Entamoeba babaci* n. sp. des Tarbagans *(Marmota babac)*. Ann. parasitol. 6 : 330-342. 1928.
21. Nöller, W. Über einige wenig bekannte Darmprotozoen des Menschen und ihre nächsten Verwandten. Arch. Schiffs-u. Tropen-Hyg. 25 : 35-46. 1921.
22. Sassuchin, D., Popoff, P., Kudrjawzew, W., and Bogenko, W. Über parasitische Infektion bei Darmprotozoen. Arch. Protistenk. 71 : 229-234. 1930.

23. SASSUCHIN, D. N. Zur Frage über die Parasiten der Protozoen. Parasiten von *Nyctotherus ovalis* Leidy. Arch. Protistenk. 64 : 61-70. 1928.
24. SASSUCHIN, D. N. Hyperparasitism in Protozoa. Quart. Rev. Biol. 9 : 215-224. 1934.
25. SPARROW, F. K. Aquatic Phycomycetes exclusive of the Saprolegniaceae and Pythium. The University of Michigan Press, Ann Arbor, Mich. 1943.
26. WENRICH, D. H. Studies on *Iodamoeba bütschlii* with special reference to nuclear structure. Proc. Am. Phil. Soc. 77 : 183-205. 1937.
27. WENRICH, D. H. Studies on the biology of *Dientamoeba fragilis*. Third Intern. Congr. Microbiol. Rept. Proc. New York. 1940. pp. 408-409.
28. WESTPHAL, A. Studien über Ophryoscoleciden in der Kultur. Z. Parasitenk. 7 : 71-117. 1934.
29. WENYON, C. M. Observations on Protozoa in the intestine of mice. Arch. Protistenk. Suppl. 1 : 169-201. 1907.
30. WINOGRADOWA, T. *Sphaerita*, ein Parasit der Wiederkäuerinfusorien. Z. Parasitenk. 8 : 356-358. 1936.

PREPARATION OF COMPLEMENT FIXING ANTIGEN FROM *CRYPTOCOCCUS NEOFORMANS*[1]

PRELIMINARY REPORT

By J. B. Fischer and N. A. Labzoffsky

Abstract

Complement fixing antigen from *Cryptococcus neoformans* strains was prepared by treating the organisms with pyridine for two hours at room temperature, washing three times with buffered 0.85% sodium chloride solution, resuspending, and subsequently exposing the suspension to ultrasonic vibration for 10 min. The antigen so prepared is not anticomplementary and appears to be specific and sensitive.

Introduction

A serologic test which would give reliable information would be an invaluable aid in the diagnosis of cryptococcal infection. It is generally recognized that the complement fixation reaction is frequently the most sensitive of the serologic procedures. To the authors' knowledge, this reaction has never been applied to cryptococcal infection.

In this preliminary report, a method of preparation of a workable and presumably specific antigen is outlined, together with the results obtained with experimental sera.

Materials and Methods

Cultures

Three strains of *Cryptococcus neoformans*, #1984, ODH127, and ODH128, were used in this study. Strain #1984, having an average cell diameter of 6.8μ and capsule thickness of 0.85μ had been recently isolated in Toronto by blood culture from a fatal case of pulmonary cryptococcosis. Strain ODH127 having an average cell diameter of 40.8μ and capsule thickness of 17μ was obtained by the courtesy of Dr. Conant of Duke University, in 1947. Strain ODH128 having an average cell diameter of 6.8μ and capsule thickness of 0.85μ was isolated from sputum of a child in Toronto, 1951. Seven-day-old cultures grown on Sabouraud agar at 37° C. were used throughout this work.

Preparation of Complement Fixing Antigen

The method of the preparation of the antigen was essentially the same as that reported for *Histoplasma capsulatum* (1) and *Mycobacterium tuberculosis* (2). Briefly, the procedure was as follows: the culture was harvested in buffered sodium chloride solution (0.85% sodium chloride containing 0.005*M* of phosphate buffer pH 7.2), washed twice with the same diluent, and after final centrifugation for 30 min. at 3000 r.p.m., the sediment was collected.

[1] *Manuscript received February 7, 1955.*
Contribution from the Central Laboratory, Department of Health of Ontario, 360 Christie Street, Toronto, Ontario.

For each 0.1 gm. of wet sediment, 5 ml. of pyridine* were added and the materials were left in contact at room temperature for two hours and shaken periodically to keep the sediment in suspension for better extraction. Next, the suspension was centrifuged and the sediment washed three times with the buffered sodium chloride solution, resuspended in the same diluent 1:20 by weight, and exposed to ultrasonic vibrations for 10 min.

Ultrasonic Generator

The ultrasonic generator model 800 obtained from Fisher Scientific Co. has a frequency of 2000 kc. and delivers 500 w. of radio-frequency energy to the transducer. To prevent the material from overheating, a special double-walled cooling vessel developed in our laboratory was used (Fig. 1). The vessel consists of an inner copper tube (*B*) closed at one end with a brass diaphragm 0.002 in. thick (*A*). The closed end screws into the base of a wider tube, which forms a jacket (*C*). During the operation, the jacket is kept half-full of dry ice. Under these conditions, the temperature of the material during vibration was kept between 12 and 17° C.

Preparation of Immune Serum

Immune serum was obtained by inoculation of normal rabbits by subcutaneous route with three weekly injections of vaccine, followed by weekly intravenous injections of live culture.

The vaccine was prepared by inactivation of the suspension of the organisms with 1% formalin (48 hr. at 4° C.), followed by centrifugation and three washings of the sediment with buffered sodium chloride solution. After the last washing the sediment was resuspended in the same diluent to a turbidity matching McFarland standard #8. The three doses administered were 0.5 ml., 1.0 ml., and 2 ml.

The viable inoculum, which was prepared fresh each week, consisted of a suspension of the organisms in buffered sodium chloride solution and standardized to the same density as the vaccine. Gradually increasing intravenous weekly doses were: 0.1 ml., 0.2 ml., 0.3 ml., 0.4 ml., and 0.5 ml.

A sample of blood was drawn from each animal prior to the immunization schedule and also before each injection and the rabbits were bled out one or two weeks after the last injection. Sera were stored at −50° C. For complement fixation test all sera were inactivated at 58° C. for 30 min.

Complement Fixation Test

Depending on whether antigen or immune serum was to be titrated, serial twofold dilutions of antigen or serum were added to constant amounts of the other ingredient and to two units of complement, each ingredient being contained in 0.2 cc. volume, using 0.85% sodium chloride as a diluent. After 75 min. at 37° C., 0.4 cc. of haemolytic system composed of 0.2 cc. of

* *Pyridine, C.P., E.A. tested purity reagent. Supplied by Fisher Scientific Co.*

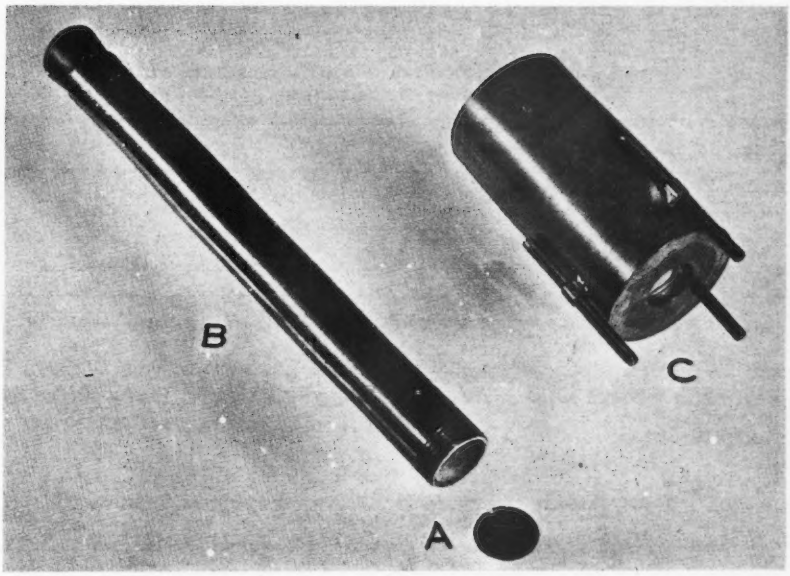


FIG. 1. Treatment vessel. *A*. Brass diaphragm. *B*. Inner tube. *C*. Cooling jacket.

2% washed sheep cells and 0.2 cc. of amboceptor solution containing two units was added. The mixture was incubated for 30 min. in the water bath at 37° C. and then read.

Results

Three different batches of antigen were prepared from the three strains which differed from each other by the thickness of the capsule. Each batch was titrated for potency, specificity, and anticomplementary activity.

In Table I results of the titrations of these antigens for potency and anticomplementary qualities are recorded. Immune serum used in these titrations was from a rabbit immunized with strain #1984.

TABLE I

TITRATION OF THREE BATCHES OF CRYPTOCOCCAL ANTIGEN AGAINST IMMUNE RABBIT SERUM

Antigen 1 in	8	16	32	64	128
Batch I, strain 1984	4+	4+	4+	3+	+
Batch II, strain ODH127	4+	4+	4+	3+	+
Batch III, strain ODH128	4+	4+	4+	2+	−

Immune rabbit serum #1984 used 1 : 20. All antigen and serum controls negative.

To obtain some idea of the antigenic similarity of the three strains, the same rabbit serum (#1984) was titrated against the three batches of antigen and the results are summarized in Table II.

TABLE II

TITRATION OF IMMUNE RABBIT SERUM #1984 AGAINST THREE BATCHES OF CRYPTOCOCCAL ANTIGEN

Serum 1 in	8	16	32	64	128
Antigen #1984 1 : 20	4+	4+	4+	4+	+
Antigen ODH127 1 : 20	4+	4+	4+	3+	+
Antigen ODH128 1 : 20	4+	4+	4+	4+	2+

All controls negative.

The results tabulated in this table appear to indicate that there is no obvious antigenic difference between the three strains tested since the titer of the immune serum from a rabbit immunized with strain #1984 was found to be practically the same in all three cases.

To determine the specificity of cryptococcal antigens, a limited number of tests was performed using *Histoplasma* and *Blastomyces* antisera. In both

cases the results were negative, indicating that there is no cross reaction between *Cryptococcus* antigen and either *Histoplasma* or *Blastomyces* antisera.

Antigen Stability

The antigen is stable, withstanding boiling in a water bath for at least five minutes without noticeable diminution in the titer.

Discussion

In the present brief report a method for the preparation of nonviable and presumably specific cryptococcal crude antigen is outlined.

No obvious difference was observed in the antigenicity of the three antigens prepared from three strains having capsules of different average thickness.

It is regrettable that no sera were available from human sources; therefore at this moment it is impossible to predict what, if any, diagnostic value the complement fixation test will have in cryptococcal infection. In the meantime, however, it offers an additional tool for studying the antigenic structure of this fungus. This work is already in progress in our laboratory.

References

1. LABZOFFSKY, N. A. Preparation of an antigen from *Mycobacterium tuberculosis* for use in the complement fixation test. To be published.
2. LABZOFFSKY, N. A. and FISCHER, J. B. Preparation of antigen from *Histoplasma capsulatum* for complement fixation test. (Abstra.) Can. J. Public Health, 46 : 47 1955.

STUDIES ON THE EFFECTS OF PHAGOCYTIC STIMULATION ON MICROBIAL DISEASE

I. ACTION OF SOME DERIVATIVES OF THE BICYCLO[0.3.5]DECAPENTAENE SKELETON ON ENDOTHELIAL CELLS OF SKIN VESSELS[1]

By Béla Gözsy and Laszló Kátó

Abstract

If the depilated skin of white mice is rubbed with solutions of histamine or certain derivatives of bicyclo[0.3.5]decapentaene, simultaneous intravenous injection of a suspension of India ink leads to an intracellular accumulation of ink particles inside the endothelial cells of small vessels at the site rubbed. This phenomenon of phagocytosis is induced in cells which have normally no phagocytic capacity and can be inhibited by antihistaminics. Solutions of these derivatives are quickly absorbed and are thought to liberate sufficient quantities of histamine to induce phagocytic activity of the endothelial cells. Some derivatives of the bicyclo[0.3.5]decapentaene skeleton have been tested for similar action and a quantitative difference was observed. These observations confirm those of Jancsó who states that histamine stimulates the endothelial cells of small vessels to phagocytic function. The phenomena described seem to have a certain importance because if the hypothesis is correct, a high number of normally inactive cells may be placed at the disposal of the natural cellular defense mechanism of the organism by means of a physiological stimulus.

Introduction

The phenomenon of phagocytosis, which is so important in normal scavenging and in combatting microbial invasion of the tissues, is imperfectly understood and, in particular, the physiological stimulus to this fundamental cellular activity is obscure. Some experiments by Jancsó (3, 4) have provided a provocative concept of the activation of the phagocytic function of cells of the reticulo-endothelial system (RES) and a starting place for our work. This paper provides confirmation of Jancsó's fundamental observation and describes a means of providing a continuing stimulation of phagocytic function in animals. Other papers in the series will show the effect of such a stimulation on microbial infection.

Jancsó's (3, 4) conclusion was that histamine is the stimulus responsible for the phagocytic function of the RES. If the depilated skin of white mice and albino rats is rubbed with a 0.5% solution of histamine and a 10% suspension of India ink is given intravenously at the same time, a black spot appears at the site of histaminization. Histological studies showed that particles of ink were found inside the endothelial cells of the small vessels. Parenteral introduction of histamine induced a general response of the organism and ink particles were found inside the endothelial cells of small vessels of the striated muscles of the intestines and lungs. This endothelial phagocytosis could be inhibited by simultaneous application of an anti-

[1] *Manuscript received November 12, 1954.*
Contribution from the Institute of Microbiology and Hygiene of the University of Montreal, Montreal, Quebec. This work was partially aided by grants from the Ministry of Health of the Province of Quebec (Federal–Provincial Health Research Grants) and from "Les Fondations Rhéaume".

histaminic substance. Jancsó demonstrated this inhibition again in the surviving, perfused liver of rats. Particles of India ink were normally retained by the Kupffer cells, but when a synthetic antihistamine was added to the liquid of perfusion the phagocytic function of these cells was completely paralyzed. A normal phagocytic function could be restored by the addition of histamine to the solution. The antihistamine inhibited a "physiological" action of histamine. It is generally admitted that endothelial cells of the small vessels do not take in particles. Jancsó proved that these mesenchymal cells may acquire such capacity under the influence of a physiological stimulus, histamine.

Jancsó concluded that histamine is not only a stimulus, which transforms the dormant endothelial cells into phagocytes, but it is also the physiological activator of the RES. In the two above mentioned experiments the antihistamine inhibited the action of histamine on the endothelial cells and the Kupffer cells as well. Jancsó explains this mechanism as due to the action of metabolite-antagonists, the antihistaminic taking the place of histamine on the specific receptor of the mesenchymal cells.

Jancsó's experiments have been confirmed by Törö (7), Biozzi, Mené, and Ovary (1), Matolcsy and Matolcsy (5). The experiments conducted by Biozzi, Mené, and Ovary (1) showed that stimuli which may liberate histamine may also induce phagocytic function of the endothelial cells of the peripheral vessels. This phenomenon can be inhibited in every case by antihistaminics and cannot be induced by vasodilator substances. Histamine is certainly the most important factor in this mechanism of defense of the organism. The latent histamine may be activated by means of several stimuli and this activated histamine induces an intensified phagocytosis.

There have been several attempts to introduce histamine for therapy. Direct application of histamine presents the serious disadvantage of rapid decomposition by the specific enzyme. Following Jancsó's experiments it seems more logical to search for histamine-activating substances for therapeutic uses. We may summarize the problem as a systematic search for such substances, which are non-toxic, have no irritating properties or no undesirable side effects, and must be slowly absorbed while continually ensuring a controlled liberation of histamine within physiological limits.

Experimental and Results

White mice of both sexes of the strain C.F. 3 weighing 20–22 gm. each have been used throughout the experiments. The abdomen of the animals was depilated by means of barium sulphide the evening before. The phagocytic reaction was provoked the next morning on animals having no lesions on the depilated spot. The histamine solution and the solutions of bicyclo[0.3.5] decapentaene derivatives were rubbed on the skin by means of a small cotton swab, gently, during two minutes. Histamine bichlorhydrate in 0.5% concentration was dissolved in 75% ethyl alcohol and the different derivatives

of the bicyclo[0.3.5]decapentaene skeleton were dissolved either in 75% ethyl alcohol or in linseed oil. The skin of animals serving as controls was rubbed with the solvents alone. Immediately after rubbing the skin, 10% India ink was injected intravenously in the tail vein, 0.5 ml. for each animal. The ink suspension contained 10% commercial India ink, 0.9% sodium chloride, and 1% gelatin, sterilized at 60° C.

Four antihistaminic substances were used: Antistin (2-phenylbenzyl-aminomethyl-imidazoline sulphate), Antergan (N-phenyl-N-benzyl-N',N'-dimethyl-ethylenediamine-chlorhydrate), Neoantergan (mepyramine maleate), and Benadryl (diphenhydramine hydrochloride).

Three different derivatives of the bicyclo[0.3.5]decapentaene (B.D.) skeleton and the natural chamazulene (1,3-dimethyl-7-ethylazulene) were used for the phagocytic studies. Each of these substances is representative of the natural and synthetic azulenes, grouped by Plattner (6) as I, II, and III by absorption in the visible spectrum.

1. From group I, natural chamazulene (1,3-dimethyl-7-ethylazulene).
2. From group II, 1,4-dimethyl-7-isopropylazulene.
3. From group II, 2-isopropyl-4,8-dimethylazulene.
4. From group III, 1,2-dimethylazulene.

The first series of experiments was intended to show the phagocytic activity of the endothelium of the small vessels as due to the application of the natural chamazulene on the skin, compared with the same phenomenon induced by histamine. Table I shows that intensive phagocytosis may be obtained by means of histamine (0.5%) or chamazulene (0.02%) if the necessary concentration of the substances has been used. Within these limits the intensity of the reaction was equal for all the animals. Where the concentration of chamazulene was lowered the reaction was proportionally lower. Specimens from two mice out of each group of four were submitted to histological studies. The phagocytic spot was cut from skin to peritoneum for this purpose.

TABLE I

PHAGOCYTIC ACTIVITY OF ENDOTHELIAL CELLS OF SMALL VESSELS INDUCED BY HISTAMINE AND CHAMAZULENE

Number of mice	Substances tested		Degree of phagocytosis 2 hr.	24 hr.
4	Histamine	0.5 %	++++	++++
4	Chamazulene	0.5 %	++++	++++
4	Chamazulene	0.05%	++++	++++
4	Chamazulene	0.02%	+++	+++
4	Chamazulene	0.01%	+	++
4	Solvents		−	−

±, +, ++, +++, ++++ = *intensity of blackening at the site of rubbing with histamine or chamazulene.*

Sections from histaminized animals or those treated with chamazulene showed no difference, owing to an intensive engulfment of carbon particles by the endothelial cells of small vessels in both cases. This intense phagocytic activity was very rapid and could be seen already after 15 min. The absorption of chamazulene was profound; even endothelial cells of vessels in the abdominal muscles and vessels of the intestines showed phagocytic activity in the animals anointed with chamazulene.

The second series of experiments was designed to show whether derivatives of bicyclo[0.3.5]decapentaene were able to induce the same phagocytosis as chamazulene or histamine. Table II shows that 1,4-dimethyl-7-isopropylazulene may activate the endothelial cells to a phagocytic function with the same intensity as the natural chamazulene.

TABLE II

PHAGOCYTIC ACTIVITY OF ENDOTHELIAL CELLS OF SMALL VESSELS INDUCED BY CHAMAZULENE AND SYNTHETIC AZULENES

Number of mice	Substances tested		Degree of phagocytosis 2 hr.	24 hr.
4	Chamazulene	0.5 %	++++	++++
4	"	0.05%	++++	++++
4	"	0.02%	++	++
4	"	0.01%	±	++
	1,4 Dimethyl-7-			
4	isopropylazulene	0.5 %	++++	++++
4	"	0.05%	++++	++++
4	"	0.02%	+++	++++
4	"	0.01%	+	+
	2-Isopropyl-4,8-			
4	dimethylazulene	0.5 %	++	++
4	"	0.05%	−	−
4	"	0.02%	−	−
4	"	0.01%	−	−
	1,2-Dimethylazulene	0.5 %	±	±
4	"	0.05%	−	−
4	"	0.02%	−	−
4	"	0.01%	−	−
4	Solvents		−	−

Azulenes of the second and third groups (Plattner) have practically no such action on endothelial cells. Only those derivatives which have no substitutes in the second and sixth position of the cycloheptane skeleton are biologically active. In this experiment, histological sections were prepared from animals which had been rubbed with a 0.02% solution of 1,4-dimethyl-7-isopropylazulene. A photograph of one of these sections is presented

PLATE I

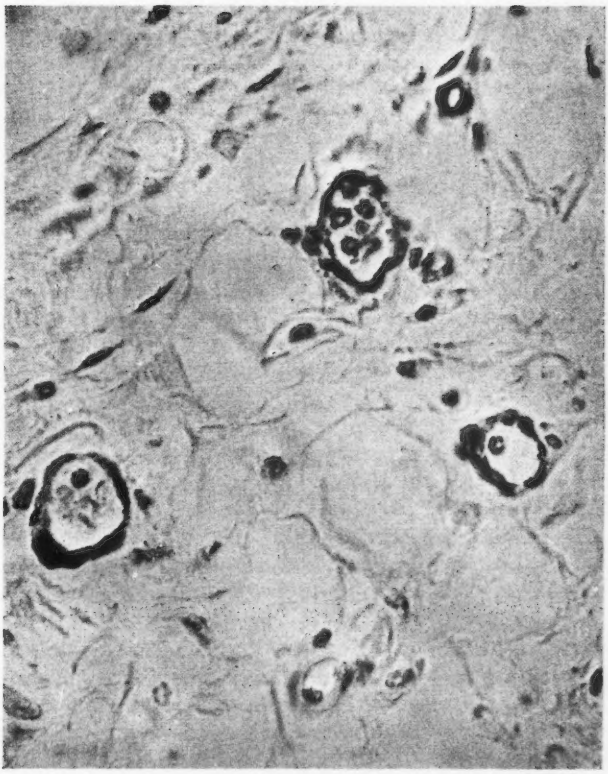


FIG. 1. Histological section of the skin showing intensive phagocytic activity of endothelial cells of the small vessels, due to the application of a 0.02% solution of 1,4-dimethyl-7-isopropylazulene on the skin.

(Fig. 1), showing the intensive phagocytic activity of endothelial cells: particles of ink are clearly seen inside the endothelial cells. A detailed histological study of this mechanism provoked by histamine was presented by Törö (7).

The third series of experiments served to show the antogonistic action of antihistaminics against the demonstrated effect of histamine, chamazulene, and 1,4-dimethyl-7-isopropylazulene. Two milligrams of each antihistaminic was injected subcutaneously in 0.5 cc. of 0.9% saline solution for each mouse 30 min. before the application of histamine or azulene. Control animals were treated with the solvent. Each of the four antihistaminics annulled the metamorphosis of endothelial cells into phagocytes and histological sections showed no phagocytic action of these cells. When the quantity of antihistaminic was diminished to 0.5 mgm. the antihistamine was not able to counteract the action of 1,4-dimethyl-7-isopropylazulene.

Discussion

The antiphlogistic effect of extracts of camomile flowers has been known for centuries. The active substance of these extracts is a blue bicyclic carbohydrate, azulene, which may be isolated from the volatile oils of *Matricaria chamomilla* and *Achillea millefolium*. The name "azulene" was given to it by S. Pierre in 1863. Azulenes are derivatives of cycloheptane, colored from violet to blue, and they are present in different volatile oils in small quantities. Heubner and Grabe (2) point out that azulene is the antiphlogistic substance in camomile oils; Jancsó (3) stated on the contrary that blue natural azulenes contained in camomile oils have histamine-liberating properties. Our experiments have confirmed again Jancsó's observations and have brought out further that synthetic derivatives of the bicyclo[0.3.5]decapentaene skeleton may induce phagocytic function of the endothelial cells of small vessels. This induced phagocytosis was of an intensity equal to that induced by histamine. In both cases antihistaminic substances antagonized and inhibited the phenomenon. In the one case, histamine itself stimulated the latent cells to a phagocytic function; in the other case, the 1,4-dimethyl-7-isopropylazulene was presumed to stimulate the production of active histamine since antihistaminic substances annulled the phenomenon. No irritating action of this derivative has been observed and large doses of the substance caused no toxic effects.

From these observations we would like to bring forth the following:

1. A cycle of defense may be supposed in which histamine plays an important role.
2. By application of a non-toxic and non-irritant derivative of the bicyclo-[0.3.5]decapentaene skeleton, a high number of inactive endothelial cells may be stimulated to help the cellular defense mechanism of the organism, thus increasing the capacity of the reticulo-endothelial system.
3. Experiments suggest that histamine activation is involved in this mechanism, but this needs further experimental evidence.

References

1. BIOZZI, G., MENÉ, G., and OVARY, Z. L'histamine et la granulo-pexie de l'endothélium vasculaire. Rev. immunol. 19 : 320-339. 1948.
2. HEUBNER, W. and GRABE, F. Uber die entzundüngswiedrige Wirkung des Kamillenöls. Arch. exptl. Pathol. pharmakol. 171. : 328-339. 1933.
3. JANCSÓ, M. Histamine as a Physiological activator of the reticuloendothelial system. Orvosok Lapja, 28 : 1025-1030. 1947.
4. JANCSÓ, M. Histamine as a physiological activator of the reticuloendothelial system. Nature, 160 : 227-228. 1947.
5. MATOLCSY, A. G. and MATOLCSY, M. The action of histamine and antihistaminic substances on the endothelial cells of the small capillaries in the skin. J. Pharmacol. Exptl. Therap. 102 : 237-249. 1951.
6. PLATTNER, PL. A. and RONIGER, H. Zur Kenntnis der Sesquiterpene. Helv. Chim. Acta, 25 : 590-594. 1942.
7. TÖRÖ, S. Histologische Untersuchungen über die Bezichungen zwischen reticuloendothelialem System und Histaminwirkung. Z. mikroskop-anat. Forsch. 52 : 552-571. 1942.

STUDIES ON THE EFFECTS OF PHAGOCYTIC STIMULATION ON MICROBIAL DISEASE

III. THE INFLUENCE OF ANTIHISTAMINES AND 1,4-DIMETHYL-7-ISOPROPYLAZULENE ON EXPERIMENTAL TUBERCULOSIS—PRELIMINARY EXPERIMENTS[1]

By Béla Gözsy and Laszló Kátó

Abstract

Albino mice were infected intravenously with *Mycobacterium tuberculosis* var. *bovis* (Ravenel strain). The animals treated with the given antihistaminic substance died significantly sooner than the non-treated control animals. In a similar experiment, the deteriorating effect of antihistamine drug on experimental tuberculosis in guinea pigs was demonstrated. On the basis of previous experiments, it is supposed that the physiological stimulation of the defense mechanism by histamine has been hampered in its function. The administration of 1,4-dimethyl-7-isopropylazulene, which is believed to be a non-toxic agent simulating histamine production, prolonged the life of infected animals. When antihistamine was given in addition to 1,4-dimethyl-7-isopropylazulene, the deteriorating effect of the antihistamine was inhibited. Results are discussed in terms of whether the host–parasite relationship can be favorably influenced by means of a stimulant of the reticulo-endothelial system.

Introduction

The experiments of Jancsó (6, 7), Biozzi *et al.* (1), Matolcsy and Matolcsy (11), and Törö (13) showed evidence that histamine is the physiological activator of phagocytosis. Endothelial cells of small vessels may acquire phagocytic activity under the influence of histamine; this phenomenon could be inhibited by synthetic antihistaminics. Kupffer cells of the surviving liver of a rat failed to show phagocytosis of particles if Antistin was added to the liquid of perfusion but a normal activity was restored by means of added histamine. Antihistaminics prolonged significantly the time of elimination of India ink from the blood vessels of rats whereas histamine had an opposite effect.

Ludany and Vajda (10) studied the same phenomenon on surviving leucocytes of rats and found that histamine increased the phagocytosis of staphylococci and typhoid bacilli while antihistaminics decreased the phagocytic activity of leucocytes *in vitro*. The action of antihistaminics on cells of the reticulo-endothelial system become even more evident from the experiments of Halpern *et al.* (3, 4, 5) who studied the anatomopathological outcome of a bacterial infection in rabbits under the influence of a synthetic antihistamine. The deteriorating effect of the given antihistaminics on the cell-linked defensive mechanism of the rabbit against typhimurium bacilli has been proved. The authors concluded that either the mobilization of leucocytes and macrophages

[1] *Manuscript received November 12, 1954.*

Contribution from the Institute of Microbiology and Hygiene of the University of Montreal, Montreal, Quebec. This work was partially aided by grants from the Ministry of Health of the Province of Quebec (Federal–Provincial Health Research Grants) and from "Les Fondations Rhéaume".

against the bacterial invaders was paralyzed by the antihistaminic substance, or the antihistamine inhibited phagocytic activity of the RES. Petry *et al.* (12) observed that in rabbits after a *Staphylococcus aureus* culture was injected subcutaneously, granulocytes occurred in great number in the early cellular infiltration instead of a normal mononuclear cell response if the animals were treated with the antihistamines. Spleens of infected and non-treated animals were quite normal. Spleens of antihistamine-treated rabbits showed a "splenitis acuta pulposa hyperplastica". These experiments demonstrated that the cellular defense mechanism of the animal is greatly affected by synthetic antihistaminics.

Gözsy and Kátó (2) have searched for synthetic compounds that would induce phagocytic function in the endothelial cells of skin capillaries and they have found that certain derivatives of bicyclo[0.3.5]decapentaene do show such a property. It was also shown (8) that non-toxic doses of 1,4-dimethyl-7-isopropylazulene (B.D. I) (the most active derivative of the bicyclo[0.3.5] decapentaene skeleton) increase the rapidity of elimination of India ink from the blood stream of rats. Further research is necessary in order to answer the following questions:

1. Could the antihistamines weaken, in the organism, the natural cellular defensive mechanism ?

2. Could the action of the natural defense mechanism in the animal be stimulated or increased by using a non-toxic stimulant of phagocytic activity during an experimental bacterial infection ?

The following working hypothesis could be formulated. If histamine really is the physiological stimulant of the RES, the evolution of an experimental infection might be favorably influenced by the activation of latent histamine. This favorable action should be antagonized by the use of antihistamines. The antihistamines themselves should aggravate the experimental tuberculosis in comparison with the non-treated animals and more so in comparison with animals treated with B.D. I, the properties of which have been mentioned (2, 8).

Experimental

Toxicity of 1,4-Dimethyl-7-isopropylazulene (B.D. I)

Twenty-five guinea pigs weighing between 350–400 gm. were divided into five groups. The animals in each group received, intramuscularly, 1, 5, 10, 25, and 50 mgm. respectively of the drug dissolved in 0.3 ml. sesame oil. The drug was administered every Monday and Thursday, for a period of 56 days. The administration of the drug produced no local necrotic reactions and no induration has been observed, except in a few animals receiving 50 mgm. doses. No weight loss was registered. On the contrary, the animals receiving 1, 5, and 10 mgm. gained weight noticeably. No death occurred during the 92 days following the last injection. Therefore the drug was well tolerated in doses up to 50 mgm. twice weekly for a period of 56 days.

Effect of B.D. I on Experimental Tuberculosis

Experiment I

Sixty male albino mice from the C.F.3 strain weighing each 20–22 gm. were used. Five mice were placed in each cage; members of each group had approximately the same weight. Forty mice were infected intravenously with 0.2 ml. of a seven-day-old culture in Dubos medium of *Mycobacterium tuberculosis* var. *bovis* (Ravenel) diluted 1:10 with phosphate-buffered saline solution. The animals were divided into three groups of 20 mice each. A non-treated infected group served as controls. A second infected group received subcutaneously 1 mgm. daily of mepyramine maleate (Neoantergan) in 0.4 ml. of saline. The third group of animals, which were non-infected, were treated in the same way as the second group. Treatment was begun on the day that the animals were infected and was continued until death. The first treatment was given immediately after infection. The course of infection was followed by daily observation of the animals and twice-weekly weighing until death. Visible lesions in the lungs were recorded at autopsy and the cumulative mortality percentage was plotted against time in days, using logarithmic probability scale paper. The method of graphic analysis described by Litchfield (9) was used as a criterion of the action of the antihistaminic substance.

Results

Fig. 1 presents graphic analysis of the time/per cent effect curves of the control group and the group of Mepyramine maleate treated mice on

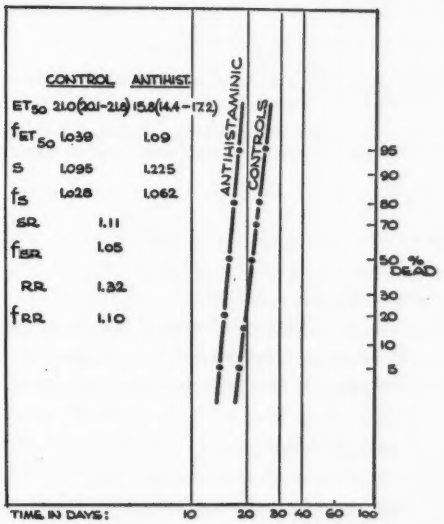


FIG. 1. Effect of Neoantergan on the mortality of mice infected with tubercle bacilli (Ravenel) as compared with the mortality of the infected non-treated control group.

logarithmic probability scale paper. The ET_{50} of control and antihistaminized groups is calculated and the reaction ratio (RR) is determined by dividing the median survival time (ET_{50}) of the control group by that of the antihistaminic treated group. S represents the slope of the time mortality lines and SR is calculated by dividing the slopes of the two groups. (RR) and (f_{RR}) are read from the nomographs of Litchfield. Since in this analysis the value of RR exceeds the value of f_{RR}, the survival time of the antihistamine treated group is significantly shorter than the survival time of the control group. Lungs of all infected animals had visible tuberculous lesions; one of the non-infected but antihistamine-treated animals died during the experiment. Loss of weight was observed during the first seven days of treatment in this latter group. Weight curves of the three groups are presented in Fig. 2.

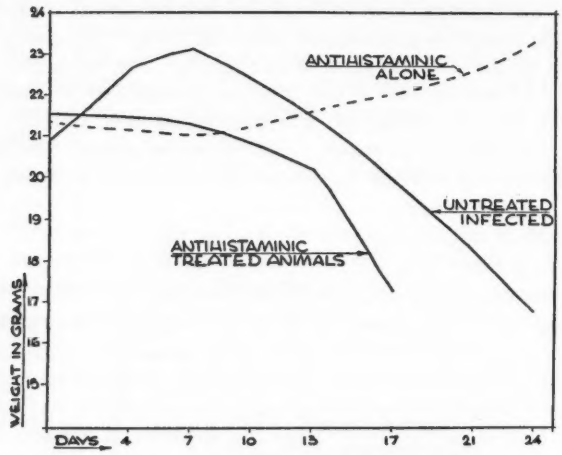


FIG. 2. Weight response curves in untreated controls and in antihistaminic-treated animals as compared with non-infected antihistaminic-treated animals.

Experiment II

Fifty-four guinea pigs, half males and half females, weighing 350–400 gm. were injected, subcutaneously, in the inguinal region with 0.1 mgm. moist weight of tubercle bacilli, Ravenel strain, obtained from a seven-day-old culture on Dubos medium. The virulence of the strain had been previously tested and the strain used was reisolated from a guinea pig. The animals were divided into six groups of eight guinea pigs each. Each group was divided into two subgroups of four animals carefully selected as to size and color and placed in two different cages. At 18 days, 46 of the 54 animals showed open lesions at the site of inoculation, and the regional lymph nodes were palpable. On the same day, six animals were killed and the pathological lesions were noted. Tuberculous lesions were found in the lungs, liver, and spleen, as well as at the site of inoculation and in the regional lymph nodes.

It was assumed that the 48 animals remaining in the experiment had tuberculous lesions comparable in extent and severity with those found in the six pretreatment controls.

The remaining six groups were treated as follows:

Group 1.— Four animals received a daily subcutaneous injection of 0.5 cc. saline solution. The other four were given, twice weekly, 0.3 ml. sesame oil, intramuscularly.

Group 2.— Daily 8 mgm. of synthetic antihistamine: diphenhydramine hydrochloride (Benadryl) in 0.5 ml. of saline solution.

Group 3.— Twice weekly, intramuscularly, 0.5 mgm. of B.D. I in 0.3 ml. sesame oil.

Group 4.— Twice weekly, intramuscularly, 2.0 mgm. of B.D. I in 0.3 ml. sesame oil.

Group 5.— Twice weekly, intramuscularly, 5.0 mgm. of B.D. I in 0.3 ml. sesame oil.

Group 6.— 8 mgm. of antihistamine as in Group 2 plus 5.0 mgm. of B.D. I as in Group 5.

In addition to these six groups, six non-infected guinea pigs, of the same weight and from one litter, received daily, from the 18th day of the experiment, an 8.0 mgm. dose of antihistamine as was done with Group 2. All animals were treated for 52 consecutive days except Sundays. The animals were allowed to survive and at death were autopsied. The pathological findings were registered and the cumulative percentage mortality was plotted against time in days of survival after infection.

In this experiment, which is considered preliminary, the Ravenel strain was used in a highly concentrated and virulent state in order to obtain quick information on the action of antihistamine and B.D. I.

Results

In all the animals of Groups 1 to 6, large nodules appeared, at the site of inoculation, as early as the 14th day. At 18 days, 46 of the animals showed ulcerations and skin perforations at the same spot. When ulceration was delayed in the other animals, it was not for more than a week. The six pretreatment controls presented open abscesses at the site of inoculation with involvement of regional lymph nodes. Visible tuberculous lesions were found in the lungs, liver, and spleen. It may be supposed that the other animals participating in the experiment would have shown lesions of the same kind and severity.

At time of autopsy, the extent of tuberculous lesions in the different groups was as follows:

Group 1.— These animals were the controls and were treated with the solvents. Within 69 days 100% died. Every animal showed large open abscesses with more or less dense discharge. The inguinal lymph nodes were

enlarged and most of them presented a necrotic caseous center. Seven animals had enlarged sacral and axillary lymph nodes with necrotic caseous centers. Three animals had enlarged inguinal lymph nodes on the side opposite to inoculation. Nodular tuberculosis with relatively severe diffuse involvement of the lungs, liver, and spleen was found in all animals. Every lymph node examined contained acid-fast bacilli.

Group 2.— These animals, which had received 8 mgm. of the antihistamine daily, died between the 20th and 48th day following infection. On the 35th day of the experiment, 50% had already died as compared with none of the control group or other groups. Antihistaminized animals showed a rapid deterioration. Loss of weight was marked after six days as compared with the controls and the non-infected antihistaminized animals. Within 5 to 10 days, the size of inguinal lesions increased greatly. For instance, in one animal the enlargement reached up to 7 × 4 cm. Axillary lymph nodes were abnormally enlarged in three out of eight animals. The autopsy of the animals showed unusually important tuberculous involvement of lymph nodes, with extensive caseous necrosis, and seldom was a lymph node found which was not filled with semisolid caseous matter. An extensive tuberculous involvement of the lungs, liver, and spleen was found in all animals. In three animals abundant pleural exudate was noted.

The animals of *Groups 3, 4, 5*, which were treated with B.D. I alone, died significantly later than the untreated animals. For instance, at 49 days, 50% of the control animals had died as compared with only 12.5% of the animals treated twice weekly with 5 mgm. of B.D. I. It is worth noting that the animals of these three groups presented relatively small pulmonary lesions. None of the axillary lymph nodes was involved or showed caseous necrosis. The inguinal lesions showed a tendency to demarcation and the suppuration was more serous than caseous.

The animals in *Group 6*, treated with the antihistamine along with the B.D. I, died at practically the same rate as the control animals. However, the macroscopic lesions resembled more those described for Groups 3, 4, and 5. None of the antihistaminized animals in the non-infected group died while the experiment lasted.

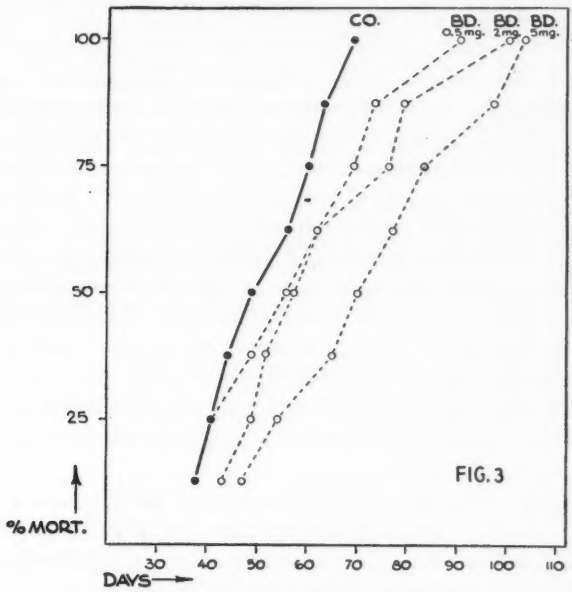
Mortality

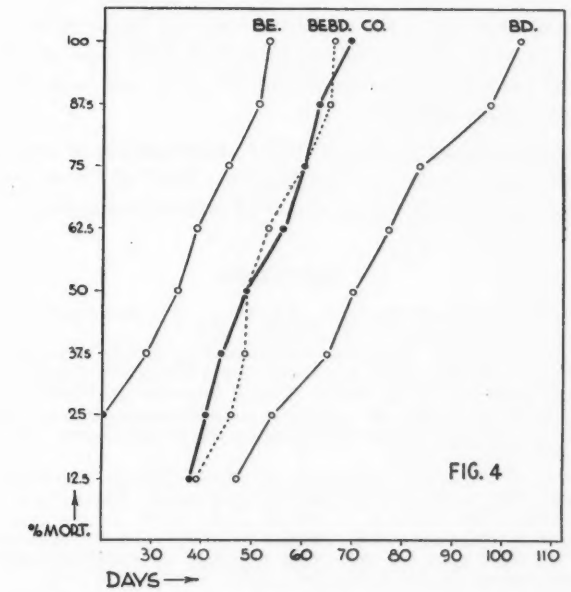
The percentage mortality for the guinea pigs participating in this experiment is shown in Figs. 3 and 4.

At 35 days: 50% of the antihistaminized animals had died and 0% of the non-treated controls.

At 53 days: 100% of the antihistaminized animals had died and 50% only of the controls; 12.5% of those treated with 5 mgm. of B.D. I had died.

At 69 days: 100% of the control animals had died and 37.5% of those treated with B.D. I (5 mgm. twice weekly).





FIGS. 3 and 4. Cumulative percentage mortality of guinea pigs infected with 0.1 mgm. tubercle bacilli (Ravenel). Treatment was begun 18 days after infection and was followed for 52 consecutive days except Sundays. CO = control animals, BD = 1,4-dimethyl-7-isopropylazulene treated animals, BE = Benadryl treated animals.

Discussion

One purpose of this study was to obtain information as to whether the host–parasite relationship could be influenced in experimental tuberculosis in mice and guinea pigs by the administration of an antihistaminic substance. Several authors have observed that the activity of cells belonging to the RES could be decreased and phagocytic function of the same cells could be partially or totally inhibited by antihistaminics *in vitro* and *in vivo*. In our experiment, the deteriorating effect of a synthetic antihistamine has been demonstrated on experimental tuberculosis in mice. This experiment brings further support that the host–parasite relationship in experimental tuberculosis is greatly dependent on the activity of the cells of the RES. A further purpose of this study was to find out if the host–parasite relationship could be favorably influenced, in the host, by a therapeutic application of a non-antibacterial agent (B.D. I) which we have found, in the course of the foregoing experiments, to be capable of inducing an increase in the phagocytic function of the RES (2, 8).

Deteriorating effects have been demonstrated on the evolution of experimental tuberculosis in guinea pigs when treated with an antihistamine. Our supposition, based on previous experiments, is that the physiological stimulation of the cellular defense mechanism by histamine has been partially paralyzed in its function by the antihistamine. On the other hand, the results show that the experimental tuberculosis in guinea pigs can be favorably influenced by means of a non-antibacterial agent, which is thought to be a histamine-activating substance.

When a given antihistamine and B.D. I are administered simultaneously to the tuberculous guinea pigs, the deteriorating effect of the antihistamine can be counterbalanced by the action of a RES-stimulating agent.

References

1. BIOZZI, G., MENÉ, G., and OVARY, Z. L'histamine et la granulopexie de l'endothélium vasculaire. Rev. immunol. 19 : 320-339. 1948.
2. GÖZSY, B. and KÁTÓ, L. Studies on the effects of phagocytic stimulation on microbial disease. I. Action of some derivatives of the bicyclo[0.3.5]decapentaene skeleton on endothelial cells of skin vessels. Can. J. Microbiol. 1 : 455-460. 1955.
3. HALPERN, B. N. and REBER, H. Action d'un antihistaminique de synthèse (3277R.P.) sur les phénomènes inflammatoires locaux d'origine microbienne. Compt. rend. soc. biol. 143 : 257-259. 1949.
4. HALPERN, B. N., DUMAS, J., and REBER, H. Rôle favorisant d'un antihistaminique de synthèse dans la généralisation de l'infection locale. Compt. rend soc. biol. 143 : 1563-1565. 1949.
5. HALPERN, B. N., BENECERAFF, B., PÉAM, V., and SALVA, J. A. Infections spontanées chez le cobaye après administrations répétées d'un antihistaminique de synthèse. Compt rend. soc. biol. 144 : 667-668. 1950.
6. JANCSÓ, M. Histamine as a physiological activator of the reticulo-endothelial system. Nature, 160 : 227-228. 1947.
7. JANCSÓ, M. Histamine as a physiological activator of the reticulo-endothelial system. Orvosok Lapja, 28 : 1025-1030. 1947.

8. KÁTÓ, L. and GÖZSY, B. Studies on the effects of phagocytic stimulation on microbial disease. II. Experiments on the physiological role of histamine in the function of the cellular defense mechanism. To be published.
9. LITCHFIELD, J. T., JR. A method for rapid graphic solution of time per-cent effect curves. J. Pharmacol. Exptl. Therap. 97 : 399-408. 1949.
10. LUDANY, G. and VAJDA, J. Die Wirkung von Histamine und Antihistaminen auf die Phagocytose der Leucocyten. Arch. intern. pharmacodynamie, 85 : 484-496. 1949.
11. MATOLCSY, A. G. and MATOLCSY, M. The action of histamine and antihistaminic substances on the endothelial cells of the small capillaries in the skin. J. Pharmacol. Exptl. Therap. 102 : 237-249. 1951.
12. PETRY, G., CSIPACK, J., KOVACS, A., and BENTZIK, M. Nauere Beiträge zur Pathologie der Pyogenen Entzundungen. I Die Rolle des Histamines beider Pyogenen Entzun dung, bzw. die Wirkung synthetischer Antihistamine auf deren Verlauf. Arch-intern. pharmacodynamie, 91 : 32-51. 1952.
13. TÖRÖ, J. Histologische Untersuchungen über die Bezichungen zwischen reticulo-endothelialem System und Histaminwirkung. Z. mikroskop-anat. Forsch. 52 : 552-571. 1942.

NOTE

EFFECT OF PHOSPHATE ON PRODUCTION OF ORGANIC ACIDS BY *ASPERGILLUS NIGER*[1]

By S. M. Martin and R. Steel

The submerged fermentation of beet sugar molasses by *Aspergillus niger* results in the production of large amounts of citric acid and smaller quantities of other organic acids (2, 4, 6, 7). Recent work (6) has shown that the ratio of citric to total acid is altered by the addition of phosphate. Since this observation suggests a marked alteration in the fermentation, an attempt has been made to identify the acids and to determine their order of appearance.

Beet sugar molasses was fermented with and without phosphate supplement (0.5 gm. $K_2HPO_4.3H_20$/liter) as described previously (6) and samples were removed at intervals for chromatographic analysis. Fermentations with added phosphate were completed in a shorter time than those without (72 hr. vs. 116 hr.). Except for this time difference, however, sugar utilization, acid production, and pH change were similar under both sets of conditions. All samples were treated with cation exchange resin (Amberlite IR100) and concentrated *in vacuo*. Samples taken after 30 hr. were treated with calcium oxide to remove most of the citric acid which otherwise interfered with the chromatograms. Samples were chromatographed in the following solvent systems: butyl acetate – acetic acid – water, 5 : 4 : 1 (5); butanol – formic acid – water, 5 : 1 : 4 (3); pyridine – ammonium hydroxide – water, 6 : 2 : 1. Developed chromatograms were sprayed with: 0.04% bromphenol blue in ethanol adjusted to pH 5.5–6.0; equal parts 0.1 *N* silver nitrate and 0.1 *N* sodium hydroxide mixed immediately before use (1); or 0.1% ninhydrin in butanol. Acids were identified primarily by comparison of R_f values in the three solvent systems. Silver nitrate and ninhydrin were used as further aids in identification. Control experiments indicated that known acids* could be detected after removal of cations. After calcium oxide treatment, however, oxalacetic, oxalic, pyruvic, and tataric acids could not be detected. Of these only the presence of oxalic acid was indicated and its concentration in the mash was estimated by calcium precipitation followed by permangate titration.

The order of appearance of the fermentation acids and an estimate of their concentrations based on spot size and density are shown in Table I. The initial mashes contained citric, glycolic, lactic, malic, and an unidentified acid (X_1) in trace amounts. Since glycolic, lactic, and X_1 acids did not appear to increase, they were not considered as products of the fermentation and

[1] *Issued as Paper No. 187 on the Uses of Plant Products and as N.R.C. No. 3608.*

* *cis-Aconitic, adipic, ascorbic, citric, fumaric, gluconic, glycolic, glutamic, 2-ketogluconic, 5-ketogluconic, α-ketoglutaric, lactic, l-malic, oxalacetic, oxalic, pyruvic, succinic, tartaric.*

are omitted from the table. When phosphate was omitted from the medium, lactic acid decreased in concentration until, at 58 hr., it could not be detected. The first acid to appear in all fermentations was 5-ketogluconic but only in those with added phosphate did its concentration increase appreciably. Phosphate had a similar effect on gluconic acid production. Under both conditions citric and malic acids began to increase at 24 to 30 hr. but of the two only citric attained a high concentration. In the absence of added phosphate, oxalic acid appeared at about 46 hr. and increased in concentration until, at the end of the fermentation, it was present in relatively high concentration. In the presence of added phosphate, oxalic appeared late in the fermentation and only in trace amounts. Traces of aconitic, fumaric, and an unknown acid (X_2) were detected late in the fermentations.

TABLE I

EFFECT OF PHOSPHATE ON PRODUCTION OF ORGANIC ACIDS FROM BEET SUGAR MOLASSES BY *A. niger*

	Sampling time, hr.							
	0	10	24	30	45	58	72	116
Without phosphate (fermentation time, 116 hr.)								
Citric	+	+	+	++	++++	++++	++++	++++
Malic	+	+	+	++	++	++	++	++
5-Ketogluconic	0	+	+	+	+	+	+	+
Gluconic	0	0	+	+	+	+	+	+
Oxalic	0	0	0	0	+	++	+++	+++
Aconitic	0	0	0	0	0	+	+	+
Fumaric	0	0	0	0	0	+	+	+
Unknown (X_2)	0	0	0	0	0	+	+	+
With phosphate (fermentation time, 72 hr.)								
Citric	+	+	++	++	++++	++++	++++	
Malic	+	+	+	++	++	++	++	
5-Ketogluconic	0	++	++	++	++	+++	+++	
Gluconic	0	0	++	++	++	+++	+++	
Oxalic	0	0	0	0	0	0	+	
Aconitic	0	0	0	0	+	+	+	
Fumaric	0	0	0	0	+	+	+	
Unknown (X_2)	0	0	0	0	+	+	+	

0 *Undetected.*
\+ *Trace (< 0.1%).*
++ *Low (about 0.2%).*
+++ *Medium (about 0.5%–1.5%).*
++++ *High (< 3.0%).*

Thus it would appear that the addition of phosphate to submerged beet molasses fermentations not only affects the yield and rate of citric acid production (6) but also alters the metabolism of *A. niger* in such a way that 5-ketogluconic and gluconic acids replace oxalic acid as important minor acidic products. This effect would appear to be a true phosphate rather than a pH effect since the addition of phosphate (0.05%) did not alter the shape of the pH curves or the final pH in the highly buffered molasses mashes.

1. BUCH, M. L., MONTGOMERY, R., and PORTER, W. L. Identification of organic acids on paper chromatograms. Anal. Chem. 24 : 489-491. 1952.
2. CLEMENT, M. T. Citric acid fermentation of beet molasses by *Aspergillus niger* in submerged culture. Can. J. Technol. 30 : 82-88. 1952.
3. LUGG, J. W. H. and OVERELL, B. T. One- and two-dimensional partition chromatographic separations of organic acids on an inert sheet support. Australian J. Sci. Research, Ser. A, 1 : 98-111. 1948.
4. MARTIN, S. M. and WATERS, W. R. Production of citric acid by submerged fermentation. Ind. Eng. Chem. 44 : 2229-2233. 1952.
5. MORTIMER, D. C. Paper chromatographic separation of some biologically important phosphate esters. Can. J. Chem. 30 : 653 -660. 1952.
6. STEEL, R., LENTZ, C. P., and MARTIN, S. M. Submerged citric acid fermentation of sugar beet molasses: Increase in scale. Can. J. Microbiol. 1 : 299-311. 1955.
7. STEEL, R., MARTIN, S. M., and LENTZ, C. P. A standard inoculum for citric acid production in submerged culture. Can. J. Microbiol. 1 : 150-157. 1954.

RECEIVED JANUARY 26, 1955.
DIVISION OF APPLIED BIOLOGY,
NATIONAL RESEARCH COUNCIL,
OTTAWA, ONTARIO.

Notes to Contributors

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Style, arrangement, spelling, and abbreviations should conform to the usage of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

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An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

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References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should be given and inclusive page numbers are required. All citations should be checked with the original articles, and each one referred to in the text by the key number.

(iv) ***Tables***

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should be used only when they are essential. Numerous small tables should be avoided.

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